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Environmental Effects of Western Coal Surface Mining. Part VII. Microbial Effect on the Quality of Leach Water from Eastern Montana Coal Mine Spoils

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Environmental Effects of Western Coal Surface Mining

Part VII. Microbial Effect on the Quality of Leach Water from Eastern Montana Coal Mine Spoils

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16. ABSTRACT

Selected portions of test cores from the overburden of the West Moorhead coal deposit in southeastern Montana were examined for possible addition to leach water of toxic substances and for the presence of iron and sulfur bacteria which might contribute to leaching. Leachates were evaluated by measuring pH, lead, and the effect of the leachates on the Selenastrum algal assay.

Both sulfur- and iron-oxidizing bacteria were isolated from a number of core samples. These bacteria differed nutritionally from thiobacilli and from other bacteria known to be involved in oxidizing sulfur and iron. Concentrations of lead in the leachates were comparable; they were not proportional to the lead content of the core samples. Most core samples were nearly neutral in reaction but some were highly acid. All acid leachates were toxic to Selenastrum. For reasons which were not determined, some non-acid leachates were also toxic to Selenastrum and some leachates stimulated Selenastrum growth.

It is concluded that acid formation in overburden spoils would be a problem only in the circumstance when a potentially acid-forming stratum is so placed during spoil reclamation that it drains directly into a surface stream. It is recommended that laboratory bioassays be used adjunct to chemical analysis for identifying problem strata.

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ENVIRONMENTAL EFFECTS OF WESTERN COAL SURFACE MINING

PART VII - MICROBIAL EFFECT ON THE QUALITY OF LEACH WATER FROM EASTERN MONTANA COAL MINE SPOILS

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FOREWORD

Some of the most severe effects of surface coal mining in eastern U.S. have been a result of leaching of mine spoils especially where oxygen is also available. If mine spoils can be properly placed to avoid such effects, the impact of mining can be reduced.

This study is one of a series to assess the environmental impact of western surface coal mining. In this report, the question of acid formation potential from mine spoils was investigated. The toxicity of leachates to algae was also assessed. While not complete enough for final answers, this study does provide useful insight into mine spoil impacts in western regions of the U.S.

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Deputy Director
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ABSTRACT

Eight test hole cores of overburden grab samples from the Bear Creek Study Site of the West Moorhead coal deposit in eastern Montana were received from the U.S. Geological Survey. The samples were visually inspected for evidence of mineralization. This was aided by an accompanying geological log for each core. Each sample was ground prior to its analysis for lead content, pH value, conductivity, and chemoautotrophic bacteria presence. The mineralization of the core samples was quantitatively different for the strata, with no consistent relation between physical and chemical descriptions.

Chemoautotrophic bacteria, both sulfur and iron oxidizing, were isolated from a number of core samples. Difficulty was encountered in obtaining pure cultures. Growth was not enhanced when organic compounds useful for some *Thiobacillus* species were included in the medium. The fastidiousness of these cultures does not typify sulfur or iron oxidizers in general, and therefore is characteristic of these isolates. By contrast, a culture of iron oxidizing bacteria isolated from a revegetation study site on the Colstrip coal deposit in eastern Montana was similar to the typical iron oxidizing bacterium *Thiobacillus ferrooxidans*.

Leaching studies were performed on samples which exhibited a wide range of lead concentrations, pH values, and conductivities. Samples were ground to <80 mesh. The study included the comparision of static and shaking conditions, with and without added glucose, with and without an inoculum of sulfur and iron oxidizing bacteria. Lead values in the leachates were roughly similar and not proportional to the lead content of the core samples. Consequently, autotrophic oxidation did not proceed at the rapid rate which occurs with unbuffered high pyrite cores. Most strata did not develop low pH values on leaching but a few strata did. Values as low as pH 1.61 were observed.

The algal bioassay procedure (U.S. EPA 1971) was used to determine the possible toxic effect of the leachates using *Selenastrum capricornutum* Printz, and a 1:10 dilution of the leachate. Growth of the algae was monitored by fluorescence spectrophotometry on a daily basis. Some leachates produced inhibition of algal growth explained by low pH values. The toxicity of other leachates could not be explained but was not due to either pH or to lead. For the majority of leachates no toxicity was observed, and some leachates were stimulatory.

CONTENTS

Foreword ii
Abstract iii
Figures v
Tables vi
Acknowledgments viii

I Introduction 1

II Conclusions 4

III Recommendations 6

IV Description of Study Area 7

V Materials and Methods 10

Media 10
Preparation of Glassware 10
Sampling 12
Sample Analysis 12
Leaching Studies 13
Algal Bioassays 16

VI Results 19

Sample Analysis 19
Enrichment Cultures 19
Algal Growth 40
Leaching Studies 42
Algal Bioassays of Leachates 62
Relation of Chemical Composition to Presence of Bacteria . . . 64

VII Discussion 68

VIII Summary 74

References 75

FIGURES

Figure		Page
1.	Map of West Moorhead, Decker, and Colstrip areas of the Fort Union coal region.	8
2.	Map of Bear Creek study area showing overburden core drilling sites.	9
3.	Algal growth curves of *Selenastrum capricornutum* Printz, comparing six methods of biomass monitoring.	41
4.	Algal growth studies of *Selenastrum capricornutum* Printz, determining the influence of five factors on algal growth, as measured by *in vivo* fluorescence method . . .	43
5.	Algal control growth curves of *Selenastrum capricornutum* Printz, used with different leachate bioassays as determined by *in vivo* fluorescence measurements	44

TABLES

Table		Page
1.	Autotrophic growth media.	11
2.	Percent of thiosulfate oxidized and resulting pH values for thiobacilli.	14
3.	Content of the leach flasks for both static and shaking conditions.	15
4.	Analysis of core sample DH75-102.	20
5.	Analysis of core sample DH75-103.	21
6.	Analysis of core sample DH75-104.	22
7.	Analysis of core sample DH75-106(A)	23
8.	Analysis of core sample DH75-106(B)	24
9.	Analysis of core sample DH76-108.	25
10.	Analysis of core sample DH75-109.	26
11.	Analysis of core sample DH76-111.	27
12.	Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-102.	28
13.	Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-103.	29
14.	Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-104.	31
15.	Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-106(A)	33

Table		Page
16.	Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-106(B)	34
17.	Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH76-108	36
18.	Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-109	37
19.	Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH76-111	39
20.	Core and leachate analyses and algal bioassays, core sample DH75-102	45
21.	Core and leachate analyses and algal bioassays, core sample DH75-103	47
22.	Core and leachate analyses and algal bioassays, core sample DH75-104	49
23.	Core and leachate analyses and algal bioassays, core sample DH75-106(A)	51
24.	Core and leachate analyses and algal bioassays, core sample DH75-106(B)	53
25.	Core and leachate analyses and algal bioassays, core sample DH76-108	56
26.	Core and leachate analyses and algal bioassays, core sample DH75-109	58
27.	Core and leachate analyses and algal bioassays, core sample DH76-111	59
28.	Results of algal bioassays of leachates of core samples following pH adjustment to pH 8.0, percent stimulation or inhibition	65
29.	Chemical analysis of overburden core samples in relation to presence of bacteria which oxidize iron and sulfur compounds	66

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SECTION I

INTRODUCTION

The replacement of a deep coal deposit and overburden by crushed overburden (spoils) in the Fort Union coal area means the replacement of a known aquifer by one with unknown characteristics. Consequently it is of interest to determine the changes in water quality that may be expected to occur from the leaching that will inevitably take place when water moves through spoils. Crushed sedimentary rock exposed to air and water is subject to bacterial leaching as well as to chemical leaching. This combination of chemical and bacterial leaching usually results in changes in total dissolved solids, in salt content, hardness, and conductivity as well as in the concentration of many metal and nonmetal elements. Bacterial leaching is most pronounced in disturbed sedimentary strata when these contain appreciable amounts of sulfides. The Fort Union coal is rated as low in sulfur. However, there are local accumulations of pyrite such that coal blending is occasionally necessary to meet the low sulfur requirement before shipping. The overburden contains highly variable but usually small amounts of sulfides. In toto, the amounts of sulfides are not great enough to presuppose an acid problem. Sulfides are frequent enough to suggest that bacterial leaching will be influenced by their presence.

The solubilization of metals from mining spoils, including coal mine spoils, has been well documented by Galbraith et al. 1972, Silverman and Ehrlich 1964, Tuovinen and Kelly 1974, and Fjerdingstad et al. 1976. The metals, as sulfides, will oxidize upon exposure to oxygen resulting in the formation of the corresponding metal sulfates, by the following reaction (Bosecker et al 1978; Fjerdingstad et al 1976; Razzell and Trussell 1963; Silverman and Ehrlich 1964; and Temple and Delchamps 1953).

$$MeS + 2O_2 = MeSO_4$$

The presence of pyrite (iron disulfide, FeS_2) in spoils causes the most vigorous solubilization of metals, as a consequence of bacterial production of ferrous, ferric, sulfate and hydrogen ions from the pyritic material (Singer and Stumm 1970; Temple and Delchamps 1953; and Temple and Koehler 1954). The ferric ion, produced through bacterial action, is a potent chemical oxidant, resulting in the solubilization of minerals (Singer and Stumm 1970; Temple and Delchamps 1953). Members of the bacterial genus *Thiobacillus* catalyze the solubilization of metal sulfides due to the general ability of the genus to oxidize reduced sulfur compounds and to the specific ability of the species, *Thiobacillus ferrooxidans*, also to oxidize reduced

ferrous iron ion (Silverman and Ehrlich 1964; Singer and Stumm 1970; Temple and Delchamps 1953; and Temple and Koehler 1954). Temple and Koehler (1954) recognized that the overall reaction sequence was a mixture of chemical and biological reactions which they expressed as:

Initial chemical reaction:

$$FeS_2 + 3\tfrac{1}{2}\ O_2 + H_2O = FeSO_4 + H_2SO_4$$

Bacterial reaction carried out by *T. ferrooxidans*:

$$2\ FeSO_4 + \tfrac{1}{2}\ O_2 + H_2SO_4 = Fe_2(SO_4)_3 + H_2O$$

Subsequent chemical reactions:

$$Fe_2(SO_4)_3 + FeS_2 = 3\ FeSO_4 + 2\ S$$

Bacterial reaction carried out by *T. thiooxidans*:

$$S + 1\tfrac{1}{2}\ O_2 + H_2O = 2\ H^+ + SO_4^=$$

According to Silverman (1967) and Beck and Brown (1968) the biological oxidation of pyrite is thought to proceed by two mechanisms concurrently: a direct contact mechanism which requires physical contact between bacteria and pyrite particles and an indirect contact mechanism according to which the bacteria oxidize ferrous ions to the ferric state, thereby regenerating the ferric ions required for chemical oxidation of pyrite. Singer and Stumm (1970) and Bosecker et al. (1978) postulate that the oxidation of metal sulfides in the presence of pyritic material is mainly restricted to the chemical oxidation of sulfides via ferric iron. They also postulate that the activity of chemoautotrophic bacteria is mainly restricted to the reoxidation of chemically reduced ferric iron. Bryner and Anderson (1957) and Brierley (1978) observed the bacterial oxidation of a metal sulfide, molybdenite (MoS_2), in the absence of pyrite. From these references, it could be assumed that oxidation of metal sulfides by chemoautotrophic bacteria will occur with or without pyrite, but that the rate of oxidation will be more rapid when associated with pyrite. Other bacteria and fungi, such as *Bacillus* species and *Penicillium*, have been shown to aid in the solubilization of metals, through the production of organic acids and some unidentified compounds (Tuovinen and Kelly 1974). However, the contribution of heterotrophic microorganisms in metal solubilization may be limited, due to low leaching yields, whereas the participation of *Thiobacillus* species in metal solubilization has been widely accepted and exploited (Tuovinen and Kelly 1974).

Visible halos are frequently seen surrounding metal ore deposits. These halos consist of dissolved and reprecipitated minerals, often acid salts, and are formed by the chemical reaction of bacterially produced sulfuric acid with carbonates found in the ore or overburden (Silverman and Ehrlich 1964). These halos are common in Fort Union coal strip mine reclaimed spoils. During rainy periods, these salts are dissolved and eventually enter the ground water.

This study on overburden leaching was initiated by the U.S. Geological Survey with cores supplied by them and was continued with support from the EPA. The EPA recommended *Selenastrum* algal assay test was used to determine toxicity of leachates.

As coal mining expands with the current and future energy demand, a more complete understanding of alkaline and acid mine drainage from coal mine spoils is required. This project studied coal mine spoils from eastern Montana which is characterized by alkaline mine drainage. The method of this project was to leach core samples under conditions in which bacterial leaching might occur and to examine the leachates for toxicity by the algal assay. Analyses for Pb were also made in the cores and their leachates, since this element is both a toxic material and a possible indication of autotrophic leaching. The purpose of these procedures was to provide a spot check on possible potential water problems which might be caused by bacterially assisted leaching of disturbed overburden.

SECTION II

CONCLUSIONS

The following conclusions are based on a limited study of individual strata from coal overburden cores. The core samples available to us were grab samples, chosen by an experienced geologist after core logging but without the benefit of mineralogical or chemical analysis. Our conclusions are indicative, but should not be considered comprehensive.

1. Leachates from the individual strata which we examined varied both in pH and their toxicity to algae from causes other than acidity.

2. Most of the strata tested produced leachates that were either basic, neutral, or weakly acidic in reaction. These leachates would not consitute an acid problem under field conditions. A few of the strata we tested produced strongly acid leachates. These acid-forming strata amount to a relatively very small part of the total overburden mass in any core.

3. The lead content of the samples which were leached did not correlate with the lead content of the leachates. Since a positive correlation is expected when lead and pyrite occur together, we conclude that there is no such association in the samples tested. Whether any other potentially toxic elements were present in the few cases of acid leachates is a question that was not examined.

4. Several leachates proved to be toxic according to the algal bioassay. A few of these were toxic because of their low pH. The toxicity of the others was confirmed by repeated experiments but the chemical basis for their toxicity was not determined.

5. The biological leaching procedure used in this study could be used to identify both acid-forming strata and toxic leachate-forming strata.

6. An extrapolation of these results to the coal field reclamation situation leads to the conclusion that both acid leachates and toxic leachates would be rarely encountered. This study was designed as an exploratory spot check. It is therefore impossible to extrapolate from it to quantitative conclusions. The overburden at the sites where our cores were taken consists of roughly horizontal strata which individually vary from less that a few centimeters to several meters in thickness, but which in total add up to between 28 and 93 meters of overburden. It is well known that any given stratum undergoes changes in chemical and mineralogical make-up over the horizontal distance of a coal field. For an area as extensive as the Fort

Union coal, these changes should be major ones. Any stratum might vary greatly in thickness, perhaps disappearing entirely in some localities, and having different chemical composition depending on the local sedimentary conditions of the basin at the time of deposition. Our spot check of selected samples from a few cores in a small part of this coal district agrees with the general opinion that Fort Union coal has for the most part an innocuous overburden. This spot check has also pointed out that some overburden strata, at some sites, are potentially toxic and that others are potentially acid-forming. A quantitative prediction about these features in the coal field as a whole requires considerable guesswork, when spot check samples are the basis for that prediction.

7. With due regard to the reservations expressed in Conclusion 6, we conclude that acid formation would be a problem only in the circumstance when a potentially acid-forming stratum is so placed during spoil reclamation that it drains directly into a surface stream. The physical effect of heavy equipment during overburden removal, stockpiling, and resurfacing is to break up the relatively soft sedimentary rocks and to produce a haphazard but real mixing of strata. This minimizes the acid potential, particularly since the greater mass of overburden consists of strata which do not produce acid and which often contain carbonate minerals capable of neutralizing acidity.

8. The same reasoning suggests the conclusion that toxicity due to materials other than acid would be rare in these coal fields. However, the existence of toxic leachates from a few samples among those tested prompts the conclusion that there may be a real risk under some circumstances. Our spot check has not adequately established the degree of this risk. We know that some companies engaged in surface coal mining in the west segregate certain strata on the basis of their chemical analyses and reserve these strata for special precautionary handling during reclamation. From our results, we conclude that toxicity testing by a bioassay is a useful adjunct to chemical analysis for identifying problem strata.

9. Although not a part of this study, other observations by us in eastern Montana coal fields have demonstrated local acid spots in surface spoils which have been reclaimed by the best current practice. In these cases, the acid leachate is neutralized by carbonate minerals in the immediate vicinity of the pyrite and does not enter the ground water supply. These acid spots are recognizable as halos of precipitated iron oxide surrounding masses of nodular pyrite, which, when tested, prove to be highly acidic. The only apparent effect is the absence of plant roots in the acid zone. Pyrite in this reclaimed coal field consists of nodular masses of pyrite mixed with other minerals including carbonates, and also as large masses of museum grade cubical crystals of apparently pure pyrite. Both types oxidize slowly. In contrast, the acid-forming samples which we tested in this study do not contain any visible pyrite but do oxidize rapidly. This is an example of the expected variability in stratum composition referred to in Conclusion 6.

SECTION III

RECOMMENDATIONS

1. Individual strata from the overburden should be tested at several sites within an operating coal field prior to overburden removal. Recommendations based on visual examination of cores by a qualified geologist or geochemist would minimize the number of samples required for analysis. Strata with a high carbonate content need not be tested for acid formation. However, acid formation potential tests should not be restricted to samples with visible pyrite.

2. Leaching tests should be used to determine acid potential. These tests are more reliable than the chemical analyses sometimes recommended because of the great difference in the rate of oxidation of different pyrite deposits. The leaching study could be quantitative as well as qualitative.

3. Leaching tests should be used to determine potential toxicity of individual strata. In addition to pointing out potentially toxic strata, leaching tests might determine whether a toxic element in a stratum, found by chemical analysis of the stratum, is actually leached.

4. Strata found to be either potentially acid-forming or potentially toxic should be segregated during overburden removal and should be deeply buried in a region with minimal underground water flow and strongly compacted during reclamation, to minimize leaching.

5. The cause of the unexplained toxicities found in this study should be ascertained.

SECTION IV

DESCRIPTION OF STUDY AREA

The study area was the Bear Creek coal field in the West Moorhead coal deposit of the Fort Union coal formation in eastern Montana (Fig. 1). Overburden cores were obtained from an area which, to date, is not being mined (Fig. 2). One overburden sample was taken from a revegetation study site, 1969-11, at Colstrip, Montana, which is also part of the Fort Union coal formation (Fig. 1). The sample was obtained from an exposed chunk of coal in the overburden which was surrounded by an acid salt halo. These halos develop from the weathering of the large numbers of nodular pyrite masses which are spread over several hectares of reclaimed spoil.

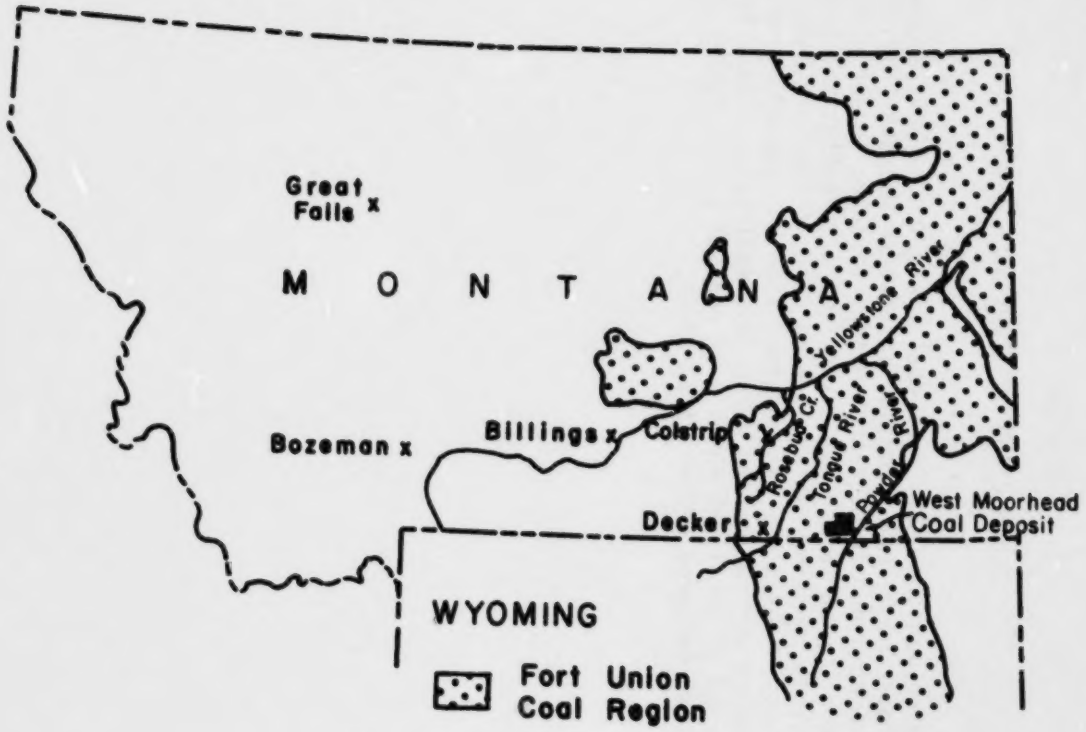

Fig. 1. Map of West Moorhead, Decker, and Colstrip areas of the Fort Union coal region. (Adapted from Van Voast and Hedges 1975.)

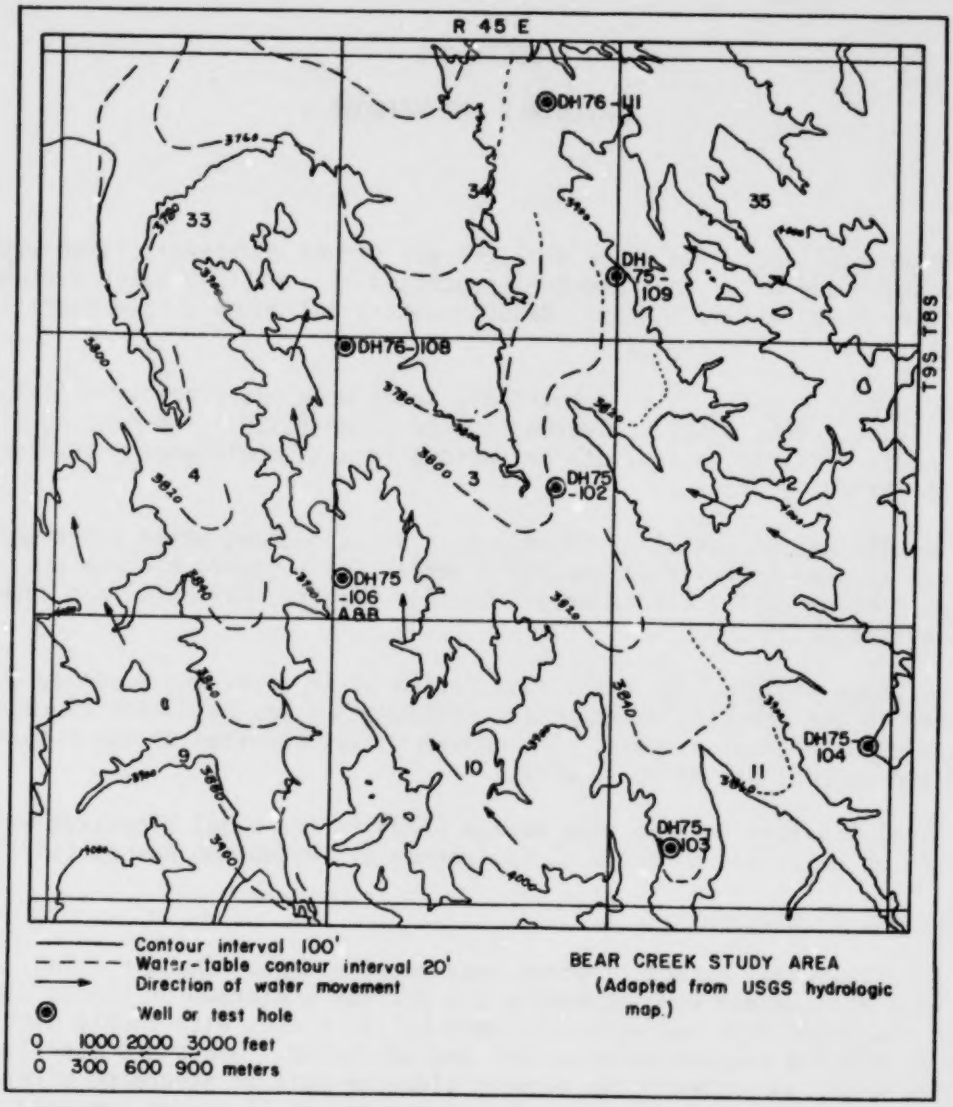

Fig. 2. Map of Bear Creek study area showing overburden core drilling sites.

SECTION V

MATERIALS AND METHODS

Media

Water utilized. The water utilized was either double-distilled water or reagent grade water that had been processed by a Milli-Q water system (Millipore Corporation, Bedford, Massachusetts) following single distillation and was stored in Pyrex glass.

Autotrophic media. The autotrophic media were described by Hutchinson et al. (1965, 1966, 1967), Silverman and Lundgren (1959), and Manning (1975). These media utilized thiosulfate or ferrous-iron as their energy sources, and are listed in Table 1.

The ISP medium was utilized only as a solid medium, while other media were utilized as both liquid and solid media. To solidify the medium, "Ionager" no. 2 (Colab Laboratories, Chicago Heights, Illinois) was added at a concentration of 1.5%.

Leachate medium. The basil salt medium of Brierley and Brierley (1973) was used in the leaching experiments. This medium was used with glucose (0.1% w/v) or without glucose. The medium pH was adjusted to the measured pH of the core sample being leached.

Algal Bioassay Medium. The medium (AAP) in the algal bioassays was prepared as described by the U.S. Environmental Protection Agency (1971).

Preparation of Glassware

All glassware, except pipettes, was machine washed and air dried. Items that were acid washed were soaked in 3 N HCl for a minimum of thirty minutes, rinsed six times with tap water followed by six rinses with double distilled water or Milli-Q reagent grade water, and air dried. Glassware sterilized by autoclaving was either covered with aluminum foil or stoppered with gauzed cotton plugs and processed for 15 minutes at 15 pounds pressure.

Pipettes were soaked in chromic acid for 30 minutes, rinsed at least fifteen times with tap water and air dried. Pipettes and glass petri dishes were placed in metal cans or boxes and oven sterilized at 177°C for 3 hours.

Table 1. Autotrophic growth media.

Reference	Media: Acid thiosulfate	Media: neutral thiosulfate	Ferrous-iron
Hutchinson et al. (1965)	S6	S5	Fe
Silverman and Lundgren (1959)			9K
Manning (1975)			ISP

Sampling

Overburden grab samples were obtained from eight test holes of the Bear Creek coal area of the West Moorhead coal field of the Fort Union coal deposit in eastern Montana by the U.S. Geological Survey. Each of the eight test hole cores was accompanied by a geological log. Each sample consisted of a portion of the test hole core, between known depths, and was placed in plastic bags for transport. The samples were stored in plastic bags at room temperature in the dark.

A sample was obtained from a revegetation study site (1969-11) at Colstrip, Montana. The sample was taken from an exposed area which exhibited evidence of iron oxidation in close association with coal distributed in the overburden. This sample was stored in a plastic bag at room temperature.

Sample Analysis

Each sample was visually inspected for evidence of mineralization. Core samples were then crushed in a mortar and pestle, analyzed for pH, conductivity, and lead, and cultured for chemoautotrophic bacteria.

pH measurement. For pH measurement, 10 g of ground sample were mixed with 20 ml of double distilled water and allowed to stand for four hours before making the pH reading with a Radiometer model 25 pH meter. Readings were stable at this time.

Conductivity measurement. Conductivity was measured by adding 10 g of ground sample to 20 ml of double distilled water of known conductivity, and allowing the suspension to stand for 4 hr before measuring. A Lab-Line Lecto model MC-1 Marke IV ohm meter was used. The listed values are therefore relative figures representing the conductivity of 20 ml of water which has been exposed to contact with 10 g of crushed sample. These values should not be considered to represent the specific values of leach water. The conductivity was recorded as μmhos/cm at 25°C.

Lead measurement. Each sample was extracted by adding 3 ml of concentrated HCl and 1 ml of concentrated HNO_3 to 1 g of ground sample in an acid washed screw capped tube. The mixture was boiled for 1-1/2 minutes prior to the addition of 3 ml of double distilled water, followed by boiling for another 1-1/2 minutes. The extract was cooled and filtered through Whatman no. 4 filter paper into an acid washed 10 ml volumetric flask. The flasks were filled to volume with double distilled water. The extracts were mixed and then stored in acid washed screw capped tubes at 4°C until analyzed. Lead was measured by atomic absorption spectrophotometry, using an Instrumentation Laboratory, Inc. model 151 atomic absorption/emission spectrophotometer. The lead concentration was reported as μg per g core sample.

Bacterial enrichment. Enrichment cultures for the chemoautotrophic bacteria, thiobacilli, were attempted with each sample. One gram of ground sample was added to each of three 125 ml erlenmeyer flasks, each flask contained one of 3 media, S6, S5, or Fe. These flasks were incubated statically at 28°C in the dark. After two weeks incubation, 0.5 ml from each flask was subcultured into 5 ml of fresh medium in capped tubes. After one month

incubation, pH and thiosulfate or ferrous-iron concentration were measured on each of the original enrichment flasks (Skoog and West 1963; Sorbo 1957). The amount of both thiosulfate and ferrous-iron oxidized was reported as a percent of the uninoculated control incubated for the same one month period. The presence of chemoautotrophic bacteria was determined from the resulting percentages and from elemental sulfur flocculation and from turbidity of the medium or from visible iron oxidation. The liquid cultures showing growth were transferred to fresh liquid medium and also plated on solid medium. When growth in the liquid medium transfers did not result, transfers were made to liquid medium (S6, S5, or Fe) which had been supplemented with yeast extract (0.05%, w/v), cysteine (10 mg/100 ml; J. A. Brierley, private communication), glutathione (10 mg/100 ml), or IM-MF additives (0.1%; Stuart et al. 1977). Liquid cultures were subsequently transferred weekly. Representative colonies were selected from the plates at weekly intervals and restreaked for purification of the culture. Strains were purified by at least three single colony isolations (Hutchinson et al. 1965). Pure cultures were characterized to species according to the scheme of Hutchinson et al. (1969) for thiobacilli taxonomy. The pH and percent thiosulfate oxidized values for thiobacilli according to the diagnostic tests of Hutchinson et al. (1969) are illustrated in Table 2.

The sample from the revegetation study site was cultured for ferrous-iron oxidizing bacteria in Fe medium. The culture was subsequently transferred to 9K medium and 9K medium plus yeast extract (0.05%, w/v), cysteine (10 mg/100 ml), glutathione (10 mg/100 ml), or IM-MF additives (0.1%). Iron oxidation and final pH were measured after incubation for 18 days.

Leaching Studies

Core sample selection. Samples were selected which exhibited a wide range of pH values, conductivity values, and lead contents.

Core sample preparation. Samples to be leached were ground and sized to <80 mesh (less than 117 μm).

Autotrophic inoculum. Autotrophic bacteria, both sulfur and iron oxidizing bacteria, were obtained from various sources. Sulfur oxidizing bacteria were isolated from the Artist's Paint Pot area and Geyser Springs, Yellowstone National Park. Iron oxidizing bacteria, isolated from the settling pond of the Decker Coal Mine, Decker, Montana, were supplied by Dr. Greg Olson. These cultures were maintained separately, and mixed prior to inoculation of the leach flasks.

Soil inoculum. A general soil inoculum was obtained from greenhouse pots immediately prior to the inoculation of the leach flasks.

Experimental leaching design. Leaching was performed in erlenmeyer flasks, under both static and shaken conditions. The shaker was a New Brunswick model VS gyrotory shaker (New Brunswick Scientific Company, New Brunswick, New Jersey) operated at a speed of 180 revolutions per minute. The flask size, the medium volume, core sample weight, and inoculum volume or weight differed for the two conditions, as shown in Table 3. Six flasks were used for each condition for each core sample. Each of the six flasks contained core sample.

Table 2. Percent of thiosulfate oxidized and resulting pH values for thiobacilli. [According to the diagnostic tests of Hutchinson et al. (1969).][a/]

Thiobacillus species	Percent thiosulfate oxidized	Resulting pH
Thiobacillus novellus	<30%	6.6-5.0
Thiobacillus denitrificans	<90%	6.6-5.0
Thiobacillus thioparus	>90%	6.6-3.5
Thiobacillus neapolitanus	>90%	3.5-2.8
Thiobacillus thiooxidans	>90%	<2.0
Thiobacillus ferrooxidans	>90%	<2.0
Thiobacillus intermedius	>90%	<2.8->2.0

[a/] Values determined from cultures grown in S6 or S5 medium for a period of 28 days.

Table 3. Content of the leach flasks for both static and shaking conditions.

Condition	Erlenmeyer flask volume (ml)	Core sample weight (g)	Medium volume (ml)	Inoculum Size	
				autotrophic (ml)	soil (g)
Static	250	5	150	1.0	5
Shaking	125	2.5	75	0.5	2.5

Three of the six flasks contained the leach medium without glucose added, while the others contained the leach medium with glucose added. The inoculum was the same for both sets of three flasks; one flask was an uninoculated sample control, one flask was inoculated with the mixture of autotrophic bacteria, and one flask was inoculated with soil from the greenhouse pots. The experiment was incubated for 30 days at room temperature in the dark.

Handling and analyses of leachates. Following the leaching period, a 15 to 20 ml portion of each leachate was removed to obtain the pH reading and lead content. Prior to lead analysis the samples were filtered through Whatman no. 4 filter paper into acid washed screw capped tubes and stored at 4°C.

The remaining leachate was used in the algal bioassays. This portion of the leachate was prefiltered (Millipore, AP25) followed by filtering through 0.45 µm membrane filter (Millipore, type HAWP) into sterile glassware. The leachate was stored in acid washed containers, either plastic bottles or glass tubes, at 4°C until the bioassay was performed.

Lead is reported as µg/g of core.

Algal Bioassays

Organism utilized. A culture of *Selenastrum capricornutum* Printz was obtained from the Environmental Protection Agency, Corvallis, Oregon.

Culture maintenance. A culture of *Selenastrum capricornutum* Printz was maintained on AAP medium (U.S. EPA 1971) by transfer of 5 ml of culture to 100 ml of fresh medium in 250 ml erlenmeyer flasks. The recommended routine stock culture transfer schedule of weekly transfers (U.S. EPA 1971) was initially decreased to 6 days, but after further experiments transfer every 2 days was found to give more reproducible assays.

Test conditions. All flasks, either for maintenance or bioassay, were incubated at room temperature under continuous cool-white fluorescent lighting at 400 ft-c (U.S. EPA 1971). The flasks were continuously shaken at approximately 100 oscillations per minute on a GIO gyrotory shaker (New Brunswick Scientific Company, Inc.). Growth curve studies were performed with 10 replicate 500 ml erlenmeyer flasks containing 200 ml of AAP medium. All other bioassays were performed in 250 ml erlenmeyer flasks containing 100 ml of AAP medium.

Preparation of inoculum. Initially, cells from the stock culture were prepared by washing as described by the U.S. Environmental Protection Agency (1971). Centrifugation was performed at 10,000 rpm for 10 minutes using a Sorval superspeed RC2-B automatic refrigerated centrifuge. After some experimentation, the inoculum was taken directly from the flasks, without washing and centrifuging.

Amount of inoculum. The starting cell concentration in the test flasks was 10^3 cells per ml (U.S. EPA 1971). The cell concentration (X) was determined from the fluorometer reading (Y) by the following equation (Fitzgerald and Uttormark 1974).

$$\frac{X}{10^3 \text{ cells/ml}} = \frac{Y}{15.4 \text{ relative fluorescence units}}$$

Preparation of glassware. Glassware was acid washed and sterilized as previously described. Test flasks were stoppered with gauzed cotton plugs.

Biomass monitoring for growth curve studies. Several methods were used to determine the biomass during the growth curve studies; these methods are subsequently described.

Turbidity. *In vivo* optical density was determined using a Varian Techtron model 635 spectrophotometer at 750 nm (U.S. EPA 1971) with a one centimeter path length in the cuvette.

Fluorescence. Fluorescence was used as one means of measuring chlorophyll, with both *in vivo* and extracted suspensions (U.S. EPA 1971). The extraction methods described by Yentsch and Menzel (1963) were followed with the following substitutions: 0.45 µm cellulose acetate membrane filter (Millipore) for glass fiber filter, sodium bicarbonate (15 mg/l) for magnesium carbonate (1 g/100 ml), 5 ml of 90% acetone for 2 ml of 90% acetone added to the grinding tube, and after grinding for 1 to 2 minutes, samples were frozen overnight prior to centrifuging instead of allowing centrifuged samples to stand for 1 to 2 hours. The tissue grinder was a Lightnin model L mixer (Mixer Equipment Company, Inc., Rochester, New York). Fluorescence was determined using a G. K. Turner Associates model no. 111 fluorometer (Palo Alto, California). Chlorophyll and phaeophytin were measured by reading the initial fluorescence (F_o), and the fluorescence (F_a) after the addition of 2 drops of 2 N HCl. Chlorophyll *a* (F_{chl}) was calculated from the formula of Yentsch and Menzel (1963).

$$F_{chl} = 1.77 (F_o - F_a)$$

Chlorophyll *a* was used in the plotting of the extracted chlorophyll data.

Absorbance. Chlorophyll was determined by absorbance using the Varian Techtron spectrophotometer at 665 nm and 750 nm (U.S. EPA 1971; Yentsch and Menzel 1963). Extraction and acidification methods were performed as for chlorophyll *a* fluorescence. Chlorophyll *a* and phaeopigment *a* were calculated according to the formula in Weber (1973) and Standard Methods (APHA et al. 1976).

$$\text{chlorophyll } a = \frac{26.7(665_b - 665_a)\ E}{VL}$$

$$\text{phaeopigment } a = \frac{26.7[1.7(665_b) - 665_a]\ E}{VL}$$

665_a = absorbance after acidification
665_b = absorbance prior to acidification
E = volume (ml) of 90% acetone added
V = volume (ml) of extract filtered
L = path length (cm) of cuvette, 1 cm

Cell count. Direct cell count was determined using a Petroff-Hausser bacterial counter (C.A. Hausser and Son, Philadelphia, Pennsylvania).

Dry weight. Dry weight and ash-free weight were determined following the methods of Weber (1973). Five ml of sample were pipetted into the tared porcelain crucible.

Biomass monitoring for bioassays. *In vivo* chlorophyll *a* fluorescence was used to monitor the biomass in the bioassay studies. The data were reported as percent inhibition, indicated by a negative value, or stimulation, indicated by a positive value, as compared to the maximum standing crop of algal control flasks. The values were calculated by the following formula.

$$X = \frac{100\ (A - B)}{B}$$

X = tabulated value, percent
A = leachate bioassay fluorescence reading
B = algal control fluorescence reading at maximum standing crop

Maximum standing crop is defined as the maximum algal biomass reached during incubation (U.S. EPA 1971).

Algal growth studies. The growth of *Selenastrum capricornutum* Printz as influences by either pH, glassware cleanliness, inoculum size, inoculum age, or inoculum washing was followed by fluorescence readings.

Medium pH. The pH of the AAP medium was adjusted with a sodium acetate-acetic acid buffer (Meynell and Meynell 1970) to five pH values, 3.6, 4.2, 4.6, 5.0, and 5.4.

Glassware acid washing. Glassware was washed with boiling chromic acid which was swirled to coat the glassware surface, rinsed 3 times with tap water, rinsed 3 times with a 3:1 concentrated HCl:concentrated HNO_3 solution, and rinsed 6 times with both tap water and double distilled water.

Inoculum size. The glassware acid washing study was run in conjunction with a study in which the inoculum size was doubled from 10^3 cells per ml to 2×10^3 cells per ml.

Inoculum age. The inoculum age was varied from the usual six-day-old inoculum to two- and four-day-old inocula.

Inoculum washing. Inoculum washing was examined by inoculating flasks with cells washed in sodium bicarbonate (15 mg/l) and with cells unwashed.

Algal bioassays of leachates. Leachate bioassays were performed using 10 ml of filtered leachate, 90 ml of AAP medium, and a volume of cells as inoculum so that the test flasks contained 10^3 cells per ml. Leachates which produced acid inhibition in the algal bioassays were reassayed after raising the pH to pH 8.0 with 1N NaOH. Fluorescence values are net values of medium plus leachate plus alga over medium plus leachate without alga.

SECTION VI

RESULTS

Sample Analysis

The diversity of the core samples can be observed from the analyses presented in Tables 4-11. Each core sample was composed of some combination of shale, sandstone, siltstone, coal, or clay. There was visual evidence of metal mineralization in some samples, exemplified by the presence of iron oxidized portions and salt crystals. Some layers of the overburden cores were composed entirely of coal, as seen in Tables 4, 5, 7, and 10, while each of the cores had coal as a part of some of their samples. The pH of most samples was above 7.0, and often as high as pH 8.8, with the highest pH value being pH 9.65 (Table 10). The majority of the samples containing coal had pH values of less than pH 5.54, but some samples with no coal also had low pH values. The sample with the lowest pH value, pH 2.31 (Table 11), contained coal along with carbonaceous shale. Lead content of the samples had a maximum of 57.0 µg per g of sample but the majority of samples had less than 20 µg/g of sample (Tables 5 and 11). The amount of lead in the sample did not appear to be related to any of the other analyses. Conductivity values ranged from the 162 µmhos/cm to 9232 µmhos/cm at 25C (Table 11), but most samples had conductivities below 3000 µmhos/cm. Conductivity values were not consistently related to either the physical composition or to the other chemical components measured, although samples with pH values less than pH 5.73 [except for DH75-106(B) sample depth 270.5-280 ft (Table 8)] also had higher conductivities than most other samples. These high conductivities ranged from 3422 µmhos/cm at 25C to the highest conductivity of 9232 µmhos/cm at 25C. Conductivities equally great also occurred with samples having pH values higher that pH 5.73.

Enrichment Cultures

Enrichment cultures for thiosulfate oxidizing bacteria in S6 and S5 media and for ferrous-iron oxidizing bacteria in Fe medium, were incubated for one month. The amount of thiosulfate or iron oxidation and the resulting pH were determined (Tables 12-19). The presence of bacteria was substantiated in many cases by wet mount microscopic examination, by observation of sulfur flocculation on the surface of the medium, or by turbidity. As Table 3 demonstrates, the different species of thiosulfate oxidizing bacteria exist over a wide pH range and oxidize variable percentages of thiosulfate. The S6 medium produced more cultures which oxidized 90% or more of the thiosulfate than did the S5 medium. The final pH values from 90% oxidation of thiosulfate in S6 medium varied from pH 6.45 to pH 2.45, which encompassed three groups of *Thiobacillus* species. The two groups which oxidize less than 90% of the thiosulfate were

Table 4. Analysis of core sample DH75-102.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm,25 C)
0- 10	Sandy clay; yellow, Fe-oxidized spots	8.15	6.8	3449
10- 20	Sandy clay	8.64	4.3	3359
20- 30	Clay; yellow, Fe-oxidized pebbles	8.43	7.5	1001
30- 40	Sandy clay, carboniferous shale; yellow, Fe-oxidized pebbles	8.49	8.2	829
40- 50	Coal	5.47	2.7	4929
50- 60	Shale and sandstone; carboniferous specks	8.46	14.4	3059
60- 70	Siltstone (light grey)	8.24	5.6	849
70- 80	Shale and siltstone	8.63	4.8	1358
80- 90	Shale; carboniferous fragments	7.29	2.8	1429
90-100	Siltstone; carbonaceous spots	7.41	4.8	1441
100-110	Siltstone; carboniferous specks	7.48	12.4	2206

Table 5. Analysis of core sample DH75-103.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0- 10	Sandy shale; Fe-oxidized spots	8.43	19.3	3133
10- 20	Sandstone; carbonaceous streaks	8.32	23.8	3703
20- 30	Shale; orange (Fe-oxidized) spots	8.14	13.2	1714
30- 40	Carbonaceous clay; coal	7.69	15.1	1803
40- 50	Layered coal	6.51	1.9	1032
50- 60	Coal	5.54	6.3	3922
60- 70	Shale	8.65	15.6	678
70- 80	Shale and sandy siltstone	7.42	7.6	1210
80- 90	Shale	8.51	10.6	986
90-100	Sandstone	8.67	4.7	772
100-110	Shaley sandstone; carbonaceous streaks	8.49	49.1	1060
110-120	Shale with carbonaceous specks and coal	6.67	21.8	1830
120-130	Sandy shale	6.23	24.3	2826
130-140	Sandstone and shale	8.59	4.7	1062
140-150	Coal and sandstone	8.92	10.3	892
150-160	Shale; carbonaceous layers	8.65	14.5	1485
160-170	Sandstone with Fe-oxidized spots; shale with carbonaceous streaks	7.91	8.6	1775
170-180	Silty clay	8.71	7.3	1191
180-188	Silty clay; carbonaceous spots	8.99	6.9	918

Table 6. Analysis of core sample DH75-104.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0 - 17	Layered, Fe layers (HCl positive), soft shale layers, sandy shale	7.44	24.2	2279
17.5- 32.8	Sandy shale; hard carbonaceous shale; clay shale	6.21	17.2	2638
37.9- 53.1	Sandy and clay shale	9.01	17.2	679
53.1- 70.9	Silty-compact shale	8.84	16.3	825
70.9- 85.2	Sandy shale, hard layered carboniferous shale	6.24	15.9	2352
85.2-100.2	Nonlayered silty and clay shale	8.84	21.2	1098
100.2-114.4	Mixed layers of clay, carboniferous shale, Fe spots	8.36	16.2	1354
114.4-131.0	Layered clay, carboniferous shale	5.73	20.0	3485
131.0-153.1	Coal and compact clay	7.61	16.4	754
153.1-169.7	Silty sandstone, crumbly coal	6.72	20.7	3065
169.7-187.1	Sandstone	8.91	17.5	1078
187.1-200.1	Sandy clay	7.06	22.8	2929
200.1-219.5	Clay, shale, and sandstone	7.15	18.9	4139
219.5-234.2	Sandy shale	8.44	13.6	1609
234.2-254.9	Compact clay, Fe spots	8.78	14.0	1482
254.9-284.8	Silt, coal	8.68	9.0	963
284.8-304.5	Siltstone, coal	6.69	6.0	3239

Table 7. Analysis of core sample DH75-106(A).

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
5-10	Yellow sand, spots of iron, dark sand in core middle	8.32	11.7	3881
10-11	Black specks (coal), may be extraneous from pulling core out; soft	8.25	11.3	3518
17-25	Natural fracture, carboniferous	8.62	7.6	2302
30-31		8.60	6.6	2215
31-32	Carboniferous material-fracture; sedimentary material with organics	8.65	7.9	1514
40	Sandstone, soft-moist	8.80	0.0	990
40-41	Carbonaceous material, soft	8.73	12.0	1372
59-60	Hard shale	8.98	7.5	874
72-73	Heavy, dense sandstone, little nonuniformity	8.99	7.6	8[illegible]
78		8.89	5.8	1064
94	Coal, moldy, supporting more life than usual	8.41	5.8	1506

Table 8. Analysis of core sample DH75-106(B).

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0 - 15	Silty shale, siltstone; Fe-oxide chunks, calcareous spots	7.2	11.6	3292
15 - 30	Silty, moist shale; Fe-oxidized silt	7.4	8.0	2170
30 - 41	Silty sandstone; Fe-oxidized silt	8.3	6.2	997
41 - 53.5	Carbonaceous shale, coal; Fe-oxidized spots	7.1	14.0	1434
53.5- 71	Shale, some carbonaceous; coal	6.7	10.0	2058
71 - 88	Shale, siltstone	8.8	17.0	720
88 -110.5	Shale, sandstone, gray claystone	9.1	15.5	858
110.5-115	Shale with Fe spots and carbonaceous spots: coal	7.9	16.5	2058
115 -133.5	Sandstone and shale; carbonaceous spots	9.1	14.2	825
133 - 146	Sandstone; carbonaceous streaks, mold	9.3	9.0	481
146 - 163	Light and gray shale, coal	7.3	14.0	2463
163 -178	Silty and coal shale, gray clay with carbonaceous spot	8.6	11.6	1341
178 -201.8	Carbonaceous shale	8.9	20.0	743
201.8-233.4	Anderson coal; salt crystals	4.2	13.0	5678
242.6-270.5	Shale with sandstone; carbonaceous streaks	8.7	11.0	582
270.5-280	Carbonaceous shale and sandstone	5.3	9.9	2463

Table 9. Analysis of core sample DH76-108.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0 - 18	Sandy clay; Fe-oxide spots (active HCl reaction), gypsum precipitate	8.46	11.3	3082
18 - 20.3	Soft sandstone	8.59	4.9	774
20.3- 43.8	Shale; Fe-oxidized, calcareous and carbonaceous spots	8.09	15.0	2122
75.8- 79.2	Very carbonaceous shale	3.43	11.5	3422
79.2- 90	Shale and sandstone	8.53	7.2	507
90 - 94.8	Shale in layers	8.07	13.3	1150
94.8-105.5	Clayey sandstone	8.27	2.8	357
105.5-138.6	Shale, carbonaceous shale, little coal	8.72	12.5	595

Table 10. Analysis of core sample DH75-109.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0 - 10	Sandy clay; tan, black carbonaceous spots, salt spots	8.59	10.1	4512
10 - 15.5	Sandy clay; black spots, salt spots, orange (Fe) deposits	8.59	12.2	3397
17 - 22.8	Sandy clay and sand; fine black, shite, and orange granules	8.54	9.5	2679
22.8- 38.9	Clay; black specks, Fe-oxide deposits, gypsum (separate bag)	7.52	15.7	3190
38.9- 60.0	Clay, coal, clinker	8.10	8.2	3014
60 -114.6	Siltstone, light shale; carboniferous spots, Fe-oxide, gypsum	6.06	8.5	2937
114.6-122.5	Coal	7.16	3.5	850
122.5-123.1	Shale; coal specks	8.59	14.4	451
123.1-142.3	Sandy siltstone	9.40	6.9	558
142.3-147.5	Shale; carbonaceous streaks	7.83	10.4	1361
147.5-149	Siltstone	9.51	8.2	472
149 -159	Shale, dark siltstone with coal deposits	8.72	10.4	840
159 -167.9	Siltstone, clay	9.46	11.1	524
170	Black shale; calcareous streaks	9.04	13.2	782
167.9-177.5	Silty siltstone and shale; black deposits	9.65	9.2	458
177.5-200	Silty siltstone and clayey siltstone	8.85	9.2	590
200 -222.2	Coal, siltstone with coal deposits	7.63	6.8	1129
222.2-230	Sandy siltstone; black deposits	8.35	6.2	784
230 -243.4	Shale; carbonaceous	8.59	8.8	709

Table 11. Analysis of core sample DH76-111.

Depth (ft)	Description	pH	Pb (µg/g)	Conductivity (µmho/cm, 25 C)
0 - 0.5	Sandy clay; roots	8.50	57.0	162
0.5- 13.4	Silty sandstone; few Fe-oxidized spots, few calcareous spots	9.24	5.5	1383
13.4- 30.5	Coal, shale; Fe-oxidized chunks and streaks	6.93	15.7	3962
30.5- 50	Clayey sandstone	8.89	9.8	1057
50 - 71.7	Carbonaceous shale	8.96	15.4	587
1C2 -105	Coal and carbonaceous shale	2.31	20.2	9232
111.5-118	Shale	9.03	12.5	932
126.5-131.7	Carbonaceous silty shale	8.48	4.8	478
131.7-156.5	Shale	8.19	17.8	1001

Table 12. Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-102.

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
0- 10	33	6.24	90	6.59	46	3.00
10- 20	62	6.14	92	5.59	34	2.96
20- 30	96	4.84	93	5.42	38	2.75
30- 40	90	5.06	95	5.50	35	2.90
40- 50	94	4.06	97	2.57	26	2.29
50- 60	94	4.57	73	4.63	16	2.74
60- 70	91	2.97	42	4.54	10	2.35
70- 80	95	2.90	26	4.49	7	2.38
80- 90	92	5.36	97	2.58	18	2.50
90-100	97	2.46	95	3.42	0	2.45
100-110	100	2.78	95	4.10	0	2.37

Table 13. Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-103.

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
0- 10	0	6.43	45	5.88	37	3.02
10- 20	100	3.14	98	4.10	27	2.95
20- 30	98	4.25	95	3.87	16	2.79
30- 40	100	2.92	97	2.87	27	2.28
40- 50	93	5.76	73	4.89	9	2.43
50- 60	88	4.98	95	4.30	4	2.40
60- 70	90	5.05	91	4.16	42	3.80
70- 80	100	2.67	4	4.63	0	2.21
80- 90	0	6.19	77	7.62	6	2.54
90-100	89	3.52	25	4.55	4	2.48
100-110	92	3.60	32	4.75	8	2.82
110-120	81	4.38	91	3.41	3	2.30

Table 13. Continued.

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
120-130	90	4.15	87	3.82	12	2.38
130-140	95	2.98	87	4.05	7	2.72
140-150	94	4.00	51	4.73	8	2.71
150-160	94	5.39	64	4.02	21	2.68
160-170	20	6.49	54	4.78	6	2.33
170-180	95	3.47	41	4.09	2	2.65
180-188	95	3.12	32	4.28	6	2.75

Table 14. Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-104.

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
0- 17	96	2.65	93	3.04	23	2.40
17.5- 32.8	92	3.99	65	3.90	11	2.53
37.9- 53.1	72	6.34	24	4.20	17	2.50
53.1-70.9	94	4.01	70	4.67	49	3.28
70.9-85.2	95	3.79	63	3.88	34	2.42
85.2-100.2	9	6.35	58	6.08	45	3.07
100.2-114.4	42	6.40	44	6.39	26	2.54
114.4-131.0	97	2.95	89	3.46	10	2.35
131.0-153.1	79	4.05	95	7.15	20	2.65
153.1-169.7	93	6.34	84	4.58	35	2.41
169.7-187.1	93	5.45	96	5.21	60	2.43
187.1-200.1	98	2.45	83	3.80	17	2.30

Table 14. Continued.

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
200.1-219.5	92	4.04	62	3.96	33	2.45
219.5-234.2	94	5.02	33	4.22	10	2.50
234.2-254.9	97	5.61	73	4.63	24	2.60
254.9-284.8	95	2.84	23	4.93	8	2.54
284.8-304.5	90	5.06	95	4.65	56	2.50

Table 15. Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-106(A).

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
5- 10	89	2.72	10	4.31	13	2.46
10- 11	93	5.64	30	4.45	42	2.90
17- 25	0	6.59	0	5.97	38	2.90
30- 31	0	6.51	0	6.02	32	3.00
31- 32	25	6.85	0	5.80	12	3.45
40	97	6.02	3	6.07	0	2.73
40- 41	97	5.56	12	6.33	5	2.83
59- 60	96	5.74	7	6.12	23	3.28
72- 73	88	5.89	29	7.40	15	3.27
78	95	5.99	6	6.00	3	3.19
94	97	5.74	15	6.56	1	3.11

Table 16. Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-106(B).

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
0- 15	97	4.95	26	6.68	6	2.75
15- 30	96	5.80	7	6.08	5	2.55
30- 41	93	4.90	3	5.73	0	2.60
41- 53.5	97	5.05	70	6.76	8	2.61
53.5- 71	82	4.70	33	4.91	24	3.26
71- 88	33	6.31	0	6.30	37	3.41
88-110.5	96	5.42	98	4.70	0	3.25
110.5-115	97	6.27	96	5.69	19	2.90
115-133.5	54	6.28	14	6.57	47	3.28
133-146	95	5.41	7	5.59	22	3.72
146-163	66	5.56	49	7.17	35	3.07
163-178	95	4.00	98	3.73	22	3.05

Table 16. Continued.

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
178-201.8	90	5.80	47	6.30	7	2.51
201.8-233.4	89	4.51	92	5.82	0	2.34
242.6-270.5	93	5.16	36	5.07	0	2.66
270.5-280	84	3.73	69	4.05	0	2.95

Table 17. Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH76-108.

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
0- 18	28	5.44	12	5.82	50	3.22
18- 20.3	96	5.61	0	5.87	5	3.34
20.3- 43.8	96	5.49	33	6.05	48	2.60
75.8- 79.2	68	7.07	74	7.03	3	2.21
79.2- 90	47	4.80	45	5.05	26	2.71
90 - 94.8	68	6.82	45	6.97	1	2.38
94.8-105.5	98	3.47	12	6.41	0	2.39
105.5-138.6	96	4.41	55	5.05	0	2.82

Table 18. Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH75-109.

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
0- 10	46	6.73	19	6.92	0	2.75
10- 15.5	18	6.71	16	6.07	33	3.05
17- 22.8	44	6.73	9	6.24	69	3.30
22.8- 38.9	95	5.35	19	6.36	0	2.49
38.9- 60.0	24	5.87	8	5.99	0	2.52
60-114.6	95	2.62	61	4.30	0	2.37
114.6-122.5	96	5.79	95	7.10	0	2.60
122.5-123.1	59	6.14	45	7.14	0	2.27
123.1-142.3	97	5.76	9	6.10	27	3.35
142.3-147.5	21	7.12	39	6.86	0	2.23
147.5-149	96	5.88	4	6.07	33	3.48
149-159	95	6.45	33	5.25	0	2.65
159-167.9	20	6.32	10	6.19	17	3.34

Table 18. Continued.

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
170	22	6.99	22	6.76	95	7.88
167.9-177.5	50	6.32	20	6.65	0	2.35
177.5-200	96	5.70	18	5.35	42	3.34
200-222.2	96	3.97	41	5.10	1	2.58
222.2-230	96	4.71	29	6.75	0	2.37
230-243.4	95	4.90	96	6.00	0	2.64

Table 19. Percent of oxidized thiosulfate and ferrous-iron, and resulting pH from enrichment cultures of core sample DH76-111.

Depth (ft)	S6		S5		Fe	
	Percent thiosulfate oxidized	pH	Percent thiosulfate oxidized	pH	Percent ferrous-iron oxidized	pH
0- 0.5	98	5.43	91	4.07	73	2.33
0.5- 13.4	86	5.87	93	5.45	28	3.18
13.4- 30.5	90	3.87	31	5.03	18	2.51
30.5- 50	92	4.95	91	4.57	39	2.95
50 - 71.7	38	5.92	35	5.55	20	2.66
102 -105	77	7.37	89	6.06	0	2.23
111.5-118	96	5.67	95	5.47	19	2.69
126.5-131.7	94	3.91	4	5.81	0	2.49
131.7-156.5	97	4.78	5	5.75	0	2.48

also represented in the S6 enrichment cultures. The S5 medium also produced representative cultures of these same groups, but the distribution was altered so that the latter two groups were more prominent. The two extremely acidophilic groups were not represented in the thiosulfate enrichment cultures, since none of the resulting pH values were less than pH 2.0. The percentage of iron oxidized did not exceed 73% when the pH remained below pH 3.0 (Table 19). One sample oxidized 95% of the iron, but had a final pH of 7.88 (Table 18). Purification of the bacteria by repeated streaking was unsuccessful due to failure of the bacteria to grow adequately on solid media.

Growth of the thiosulfate oxidizing bacteria was seen on subsequent transfer of the liquid culture to fresh liquid medium, as well as on transfer by streak plating of the liquid culture to solidified thiosulfate medium. Growth was also seen when single, well-isolated colonies from the solid medium were streaked on fresh solid medium. The subsequent re-streaking of single, well-isolated colonies for purification resulted in the development of multiple colony types, which occurred repeatedly with numerous attempts to purify cultures by streak plate. When single, well-isolated colonies were transferred to 5 ml of liquid thiosulfate medium in tubes, no growth took place. The S6 and S5 media were supplemented with yeast extract, but this did not enhance the development of cultures in liquid medium after growth on solid medium.

Iron oxidizing bacteria grew in the initial enrichment medium, which also contained the core sample, but growth on subsequent transfers decreased until the cultures finally failed to transfer to fresh liquid medium. Yeast extract, cysteine, glutathione, or IM-MF additives were included in some Fe media in an attempt to enhance the growth of the cultures, but the cultures again failed to grow. Purification of the cultures failed due to the inability of cultures to grow on the solid ISP medium, even after prolonged incubation.

An iron oxidizing bacterial culture isolated from the revegetation site (1969-11) at Colstrip, Montana was successfully subcultured on 9K or Fe medium. This isolate also grew in 9K medium supplemented by yeast extract, cysteine, or glutathione although these growth additives were not required. No growth occurred when IM-MF additives were included in the medium. The culture oxidized 99% of the ferrous iron and produced a final pH of 1.84.

Algal Growth

Algal growth curves. Figure 3 shows the algal growth curves of *Selenastrum capricornutum* Printz determined prior to the algal bioassays of leachates. Parameters of algal growth were: *in vivo* chlorophyll *a* fluorescence, extracted chlorophyll *a* fluorescence, extracted phaeopigment *a* absorbance, turbidity, cell count and ash free weight. *In vivo* fluorescence indicated a maximum standing crop on day 5. Extracted chlorophyll *a* fluorescence and absorbance both reached maximum values on day 4. Cell number and turbidity curves showed a similar rise, but failed to reach a maximum. Extracted phaeopigment *a* absorbance and ash free weight did not follow the growth of the algal culture.

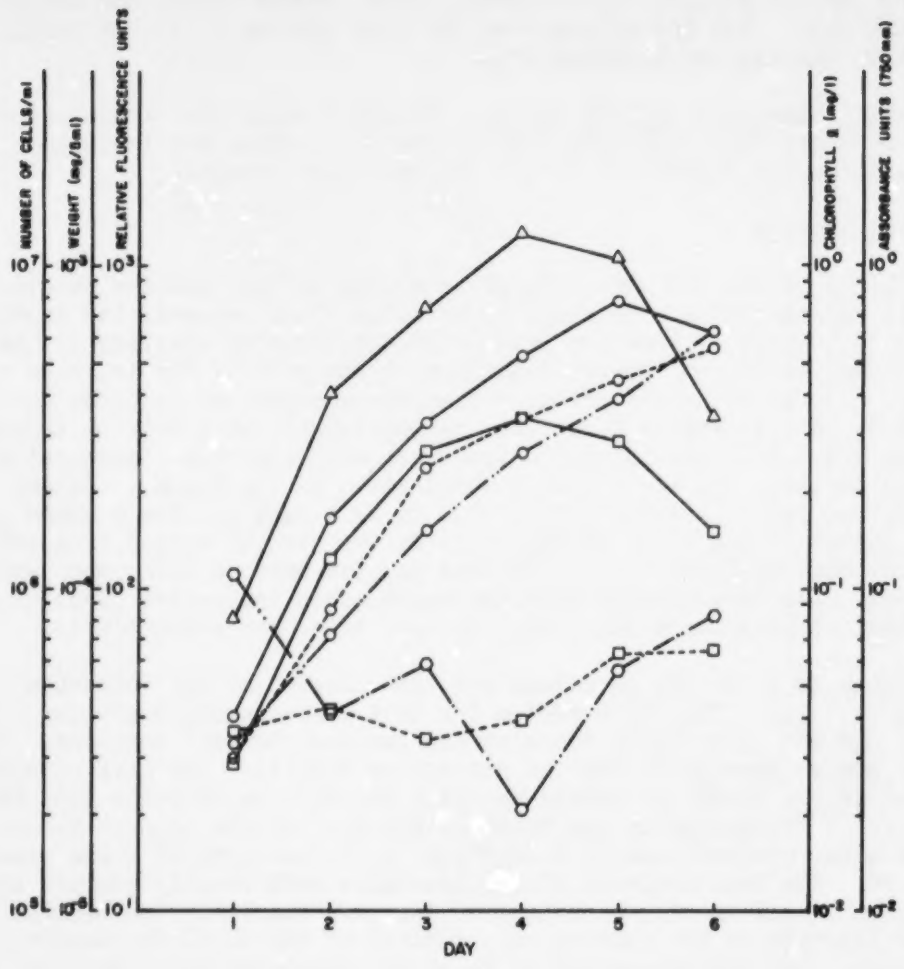

Fig. 3. Algal growth curves of *Selenastrum capricornutum* Printz, comparing six methods of biomass monitoring. [*In vivo* chlorophyll *a* fluorescence (o——o), extracted chlorophyll *a* fluorescence (□——□), extracted chlorophyll *a* absorbance (A_{665}, Δ——Δ), extracted phaeopigment *a* absorbance (A_{665}, o- - -o), turbidity (A_{750}, o---o), cell count (o——o), and ash free weight (□---□).]

Algal growth studies. Algal growth studies to determine the influence of pH, glassware acid washing, inoculum size, inoculum age, and inoculum washing are illustrated in Figure 4. In the pH study, a six-day-old inoculum was used. All acid pH values (3.6, 4.2, 4.6, 5.0, and 5.4) completely inhibited the alga. Using a six-day-old culture as inoculum, the standing crop obtained was not increased by doubling the inoculum size. Two- or four-day-old inoculum gave a greater maximum standing crop than a six-day-old inoculum. Two- and four-day-old inocula produced approximately equal growth curves and maximum standing crop. The EPA recommended inoculum washing (U.S. EPA 1971), had no effect on the maximum standing crop.

Algal bioassays control curves. Figure 5 shows the irregularity in the algal controls when a six-day-old culture was used as the inoculum. Two-day-old cultures consistently produced and increased biomass.

Leaching Studies

Thirty of the 110 core samples were used in the leaching studies. These samples represented a wide range of pH value, lead content, and conductivity. Tables 20 through 27 show the results of the chemical analyses of the core samples and leachates and the fluorometric analysis of the leachate algal bioassays. The pH values of the core samples selected for leaching studies ranged from 2.31 to 9.3 (Tables 27 and 24, respectively), with most pH values between 5.3 and 8.5. All lead values in leachates represent the concentration in µg/g of core leached, not the actual concentration in the leach solution. Most core samples had lead concentrations of 9.0 to 20.7 µg/g and the maximum was 57.0 µg/g (Tables 23 and 27). Conductivity of the samples ranged from 162 µmhos/cm to 9232 µmhos/cm (Table 27), with most samples between 1372 µmhos and 3962 µmhos/cm. The core samples selected encompassed the entire range of each of the chemical parameters analysed, viz. pH, lead, and conductivity.

Table 20 shows the pH values and lead content of the leachates from core sample DH75-102. The leach medium for this core contained glucose. Two flasks had the autotrophic inoculum and two had the soil inoculum. The recorded values are an average of the two respective results. The shaking autotrophic control of the 40-50 ft. sample caused a decrease in pH below 4.0, which was arbitrarily designated as the boundary between low and high pH values. Some of the other controls caused a decrease in pH, but none of these resulted in a low pH. The lead contents of the leachates were roughly similar and were not proportional to the Pb content of the core samples. The highest Pb was in the leachate of the shaking soil control of the 50-60 ft. sample, and was 4.65 µg/g. The average amount of Pb in the leachates was 2.60 µg/g, with the stationary leachates averaging 2.07 µg/g and the shaking leachates averaging 3.13 µg/g. The Pb values grouped according to inoculum were: uninoculated 2.16 µg/g, autotrophic inoculum 2.52 µg/g, and soil inoculum 3.12 µg/g. Stationary and shaking conditions were: stationary uninoculated 1.86 µg/g, stationary autotrophic inoculum 1.92 µg/g, and stationary soil inoculum 2.40 µg/g, and shaken uninoculated 2.46 µg/g, shaken autotrophic inoculum 3.09 µg/g, and shaken soil inoculum 3.84 µg/g.

Table 21 shows the results of the leaching of DH75-103. The pH values and Pb content of the leachates varied relatively little, with no leachates below pH 4.0 and with Pb concentrations which were proportional to the Pb

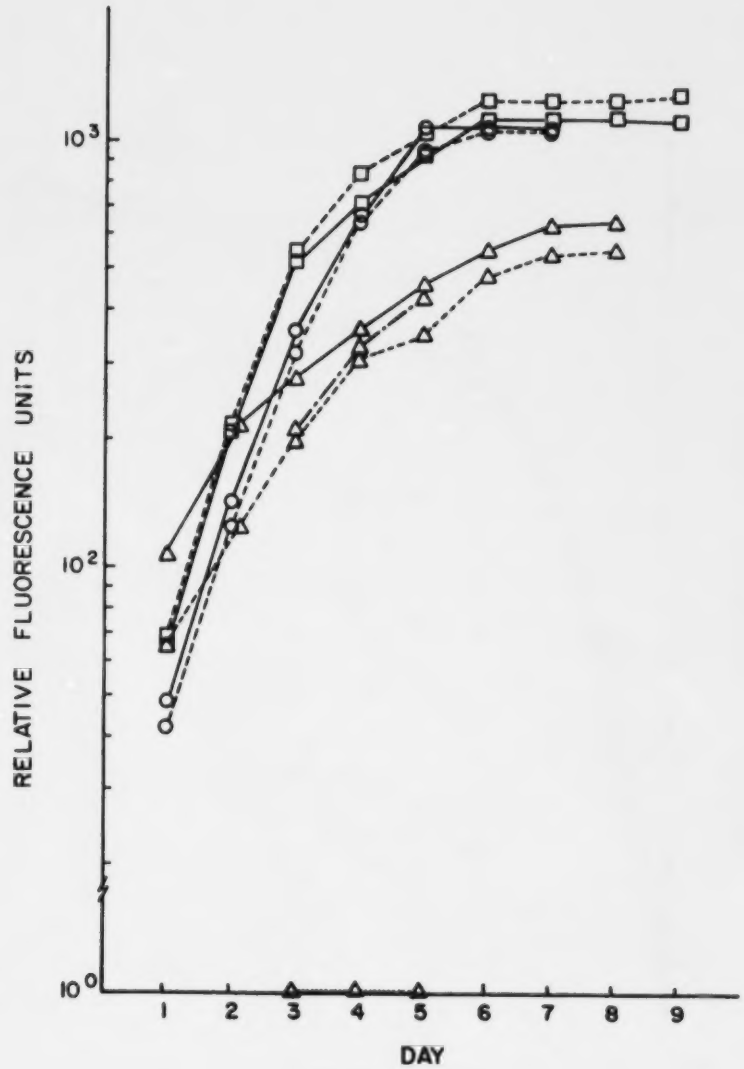

Fig. 4. Algal growth studies of *Selenastrum capricornutum* Printz, determining the influence of five factors on algal growth, as measured by *in vivo* fluorescence method. [Two-day-old inoculum (□): washed inoculum (□---□) and unwashed inoculum (□——□); four-day-old inoculum (○): washed inoculum (○---○) and unwashed inoculum (○——○); and six-day-old inoculum (△): 1X inoculum (△---△) and 2X inoculum (△——△) in chromic-HCl-HNO_3 acid washed glassware. AAP medium at pH 3.6, 4.2, 4.6, 5.0, and 5.4 (△···△) and AAP medium at pH 7.6 (△—·—△).]

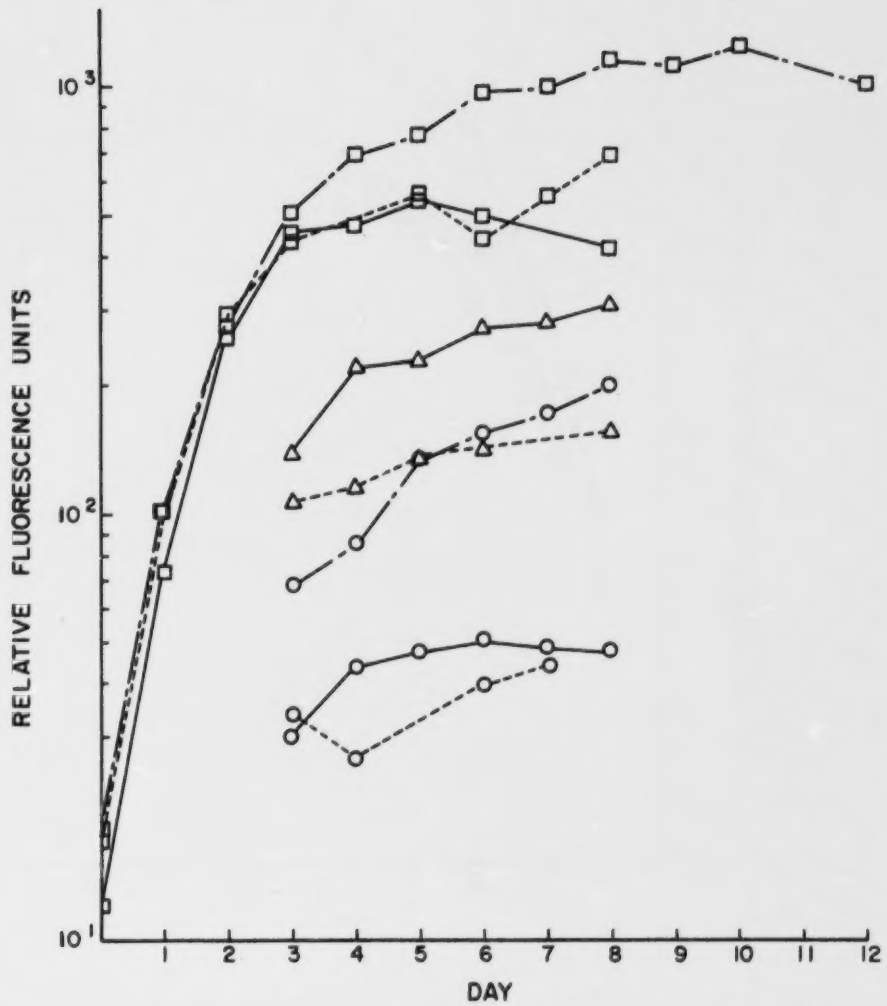

Fig. 5. Algal control growth curves of *Selenastrum capricornutum* Printz, used with different leachate bioassays as determined by *in vivo* fluorescence measurements. [Six-day-old inoculum (o and Δ): 12-5-76 (o——o), 1-8-77 (Δ——Δ), 2-16-77 (o---o), 3-25-77 (Δ---Δ), 4-29-77 (o—-—o); and two-day-old inoculum (□): 1-21-78 (□——□), 1-31-78 (□---□), and 2-11-78 (□—-—□).]

Table 20. Core and leachate analyses and algal bioassays, core sample DH75-102.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays[a] Static Leachate Percent[b]	Algal Bioassays[a] Shaking Leachate Percent[b]
40- 50	5.47	2.70	Uninoculated	With	5.80	1.50	4.83	2.40	498	-86
			Autotrophic inoculum[c]		5.72	1.50	2.90	1.95	818	-98
			Soil inoculum		5.06	2.10	4.43	2.70	586	308
50- 60	8.46	14.40	Uninoculated	With	8.35	1.50	8.02	2.40	39	418
			Autotrophic inoculum		8.26	2.10	7.89	3.45	-12	8
			Soil inoculum		6.97	2.40	5.91	4.65	-14	22
80- 90	7.29	2.80	Uninoculated	With	8.01	2.40	6.41	1.80	715	535
			Autotrophic inoculum		7.67	1.80	5.59	3.30	0	249
			Soil inoculum		6.09	2.40	5.71	4.20	-4	43
90-100	7.41	4.80	Uninoculated	With	8.03	2.10	7.83	2.70	0	51
			Autotrophic inoculum		8.06	2.10	7.35	3.75	373	469
			Soil inoculum		6.15	2.25	5.77	4.05	16	35

Table 20. Continued.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays: Static Leachate Percent	Algal Bioassays: Shaking Leachate Percent
100-110	7.48	12.40	Uninoculated	With	7.92	1.80	6.24	3.00	49	-27
			Autotrophic inoculum		7.66	2.25	5.70	3.00	198	4
			Soil inoculum		5.66	2.85	6.37	3.60	10	20

[a]Inhibition is indicated by a negative value, whereas stimulation is indicated by a positive value.

[b]Inoculum taken from a six-day-old culture.

[c]Autotrophic inocula are a mixture of sulfur and ferrous iron oxidizing bacteria.

Table 21. Core and leachate analyses and algal bioassays, core sample DH75-103.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays[a] Static Leachate Percent[b]	Algal Bioassays[a] Shaking Leachate Percent[b]
10- 20	8.32	23.8	Uninoculated	Without	6.19	2.10	6.91	1.80	-9	6
			Autotrophic inoculum[c]		6.71	1.50	7.16	1.80	-47	31
			Soil inoculum		6.17	2.10	7.48	1.80	-35	28
			Uninoculated	With	8.59	2.70	6.06	2.40	21	79
			Autotrophic inoculum		8.65	2.40	6.92	2.10	162	44
			Soil inoculum		8.21	2.70	7.24	2.10	-30	12
100-110	8.49	49.1	Uninoculated	Without	5.65	3.00	7.83	2.10	-30	618
			Autotrophic inoculum		7.25	3.30	7.96	2.70	-17	188
			Soil inoculum		6.37	3.00	7.96	3.60	-55	83
			Uninoculated	With	8.18	3.00	5.92	1.50	3	169
			Autotrophic inoculum		8.17	3.00	6.18	1.80	-10	102
			Soil inoculum		7.45	3.60	6.89	2.70	-62	79

Table 21. Continued.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays[a] Static Leachate Percent[b]	Algal Bioassays[a] Shaking Leachate Percent[b]
120-130	6.23	24.3	Uninoculated	Without	6.88	3.00	7.36	3.00	-25	-10
			Autotrophic inoculum		6.85	3.90	6.67	3.60	-10	73
			Soil inoculum		5.23	4.05	7.06	3.90	-37	60
			Uninoculated	With	6.83	3.90	6.97	2.40	6	51
			Autotrophic inoculum		6.54	4.05	6.68	3.90	-0.3	62
			Soil inoculum		6.70	4.50	6.59	3.90	11	27

[a]Inhibition is indicated by a negative value, whereas stimulation is indicated by a positive value.

[b]Inoculum taken from a six-day-old culture.

[c]Autotrophic inocula are a mixture of sulfur and ferrous iron oxidizing bacteria.

Table 22. Core and leachate analyses and algal bioassays, core sample DH75-104.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays[a] Static Leachate Percent[b]	Algal Bioassays[a] Shaking Leachate Percent[c]
0- 17	7.44	24.2	Uninoculated	Without	7.63	1.50	6.62	1.50	-9	144
			Autotrophic inoculum[d]		7.71	1.50	6.15	1.80	25	6
			Soil inoculum		6.57	1.80	7.05	2.10	-27	15
			Uninoculated	With	7.80	2.10	5.95	4.65	-67	83
			Autotrophic inoculum		7.57	2.40	6.87	2.40	-10	31
			Soil inoculum		6.74	2.70	7.44	2.10	-30	-7
114.4-131.0	5.73	20.0	Uninoculated	Without	3.22	3.90	3.25	3.30	-95	-99
			Autotrophic inoculum		3.17	2.40	2.54	4.80	-95	-99
			Soil inoculum		4.01	2.10	7.04	4.20	-96	-34
			Uninoculated	With	3.31	2.40	2.89	5.10	-95	-99
			Autotrophic inoculum		3.31	2.10	2.56	4.80	-96	-99
			Soil inoculum		4.92	1.80	7.17	3.66	-93	-58

Table 22. Continued.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays[a] Static Leachate Percent[b]	Algal Bioassays[a] Shaking Leachate Percent[c]
153.1-169.7	6.72	20.7	Uninoculated	Without	7.35	1.20	7.41	2.70	58	-78
			Autotrophic inoculum		7.36	1.80	7.49	3.30	46	-96
			Soil inoculum		6.13	2.40	7.16	2.40	8	-77
			Uninoculated	With	8.06	1.80	7.70	3.00	139	-69
			Autotrophic inoculum		8.17	2.10	7.60	3.60	50	-77
			Soil inoculum		7.33	2.40	7.46	3.30	54	-70
200.1-219.5	7.15	18.9	Uninoculated	Without	6.97	0.60	7.07	1.50	-9	-82
			Autotrophic inoculum		7.02	0.90	7.35	2.10	31	-83
			Soil inoculum		5.33	0.90	6.85	2.70	45	57
			Uninoculated	With	8.19	1.50	7.67	2.10	61	-83
			Autotrophic inoculum		8.05	2.10	7.22	1.50	46	-79
			Soil inoculum		7.90	2.10	6.87	2.70	20	45

[a]Inhibition is indicated by a negative value, whereas stimulation is indicated by a positive value.

[b]Inoculum taken from a six-day-old culture.

[c]Inoculum taken from a two-day-old culture.

[d]Autotrophic inocula are a mixture of sulfur and ferrous iron oxidizing bacteria.

Table 23. Core and leachate analyses and algal bioassays, core sample DH75-106(A).

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays[a] Static Leachate Percent[b]	Algal Bioassays[a] Shaking Leachate Percent[c]
5- 10	8.32	11.7	Uninoculated	Without	7.78	0.90	7.76	2.70	85	-77
			Autotrophic inoculum[d]		7.75	1.50	7.62	3.00	-57	-85
			Soil inoculum		5.51	2.10	6.85	3.90	335	48
			Uninoculated	With	8.16	1.80	8.28	2.70	68	-83
			Autotrophic inoculum		8.04	2.10	8.11	2.70	205	-83
			Soil inoculum		6.96	2.40	7.07	3.60	95	51
10- 11	8.25	11.3	Uninoculated	Without	8.08	0.90	8.09	0.60	-10	88
			Autotrophic inoculum		8.09	0.90	8.00	0.60	8	68
			Soil inoculum		6.04	1.50	7.06	1.50	-63	86
			Uninoculated	With	8.58	1.20	8.40	3.60	650	-81
			Autotrophic inoculum		8.52	1.80	8.64	3.60	-90	0
			Soil inoculum		7.89	1.50	7.39	1.50	30	54

Table 23. Continued.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays Static Leachate Percent	Algal Bioassays Shaking Leachate Percent
40	8.80	0.00	Uninoculated	Without	8.01	0.60	8.14	2.10	43	297
			Autotrophic inoculum		7.99	0.60	8.22	0.90	2	176
			Soil inoculum		5.42	0.90	7.07	1.50	18	-17
			Uninoculated	With	8.39	1.20	8.17	1.20	250	-1
			Autotrophic inoculum		8.33	0.90	8.35	3.00	48	208
			Soil inoculum		8.16	1.20	5.82	4.20	70	-1
40- 41	8.73	12.0	Uninoculated	Without	7.98	0.90	8.11	1.20	79	-62
			Autotrophic inoculum		8.03	1.80	6.92	1.20	44	146
			Soil inoculum		5.30	1.80	7.99	1.50	53	114
			Uninoculated	With	8.20	1.50	8.39	0.90	33	-91
			Autotrophic inoculum		8.13	1.80	8.41	0.90	7	241
			Soil inoculum		7.28	1.80	7.22	0.90	-67	-86

[a] Inhibition is indicated by a negative value, whereas stimulation is indicated by a positive value.

[b] Inoculum taken from a six-day-old culture.

[c] Inoculum taken from a two-day-old culture.

[d] Autotrophic inocula are a mixture of sulfur and ferrous iron oxidizing bacteria.

Table 24. Core and leachate analyses and algal bioassays, core sample DH75-106(B).

Depth (ft)	Sample		Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate		Shaking Leachate		Algal Bioassays[a]	
	pH	Pb (µg/g)			pH	Pb (µg/g)	pH	Pb (µg/g)	Static Leachate Percent[b]	Shaking Leachate Percent[b]
71-88	8.80	17.0	Uninoculated	Without	7.10	3.60	6.60	1.80	16	-99
			Autotrophic inoculum[c]		8.47	4.80	7.21	3.00	14	68
			Soil inoculum		5.85	5.40	7.98	2.70	14	17
			Uninoculated	With	8.80	4.50	8.26	2.40	32	-9
			Autotrophic inoculum		8.61	4.20	5.75	4.50	29	51
			Soil inoculum		7.61	3.00	8.34	2.40	29	48
133-146	9.30	9.00	Uninoculated	Without	8.43	3.00	8.41	0.00	75	-4
			Autotrophic inoculum		8.32	2.70	7.65	0.00	19	38
			Soil inoculum		5.92	4.20	6.91	0.00	83	-62
			Uninoculated	With	8.69	3.00	8.06	0.00	29	-83
			Autotrophic inoculum		8.61	2.70	7.82	3.00	42	28
			Soil inoculum		7.47	3.00	8.21	3.00	53	-98

Table 24. Continued.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays Static Leachate Percent	Algal Bioassays Shaking Leachate Percent
178-201.8	8.90	20.0	Uninoculated	Without	6.04	3.00	6.22	3.60	46	-49
			Autotrophic inoculum		5.87	1.50	6.14	3.60	-1	-35
			Soil inoculum		5.26	3.60	5.75	3.30	66	-84
			Uninoculated	With	8.07	1.50	7.72	2.70	66	-1
			Autotrophic inoculum		7.62	2.10	3.53	5.40	-7	-62
			Soil inoculum		6.43	2.70	7.49	2.70	55	42
201.8-233.4	4.20	13.0	Uninoculated	Without	2.83	3.00	3.18	4.50	-99	-100
			Autotrophic inoculum		2.35	0.00	2.31	6.00	-99	-100
			Soil inoculum		2.31	2.10	4.41	3.00	-99	-98
			Uninoculated	With	2.77	6.60	3.91	3.00	-100	-59
			Autotrophic inoculum		2.36	5.10	2.75	4.50	-99	-98
			Soil inoculum		2.45	6.00	3.94	3.00	-99	-14

Table 24. Continued.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays: Static Leachate Percent	Algal Bioassays: Shaking Leachate Percent
270.5-280	5.30	9.90	Uninoculated	Without	7.08	1.20	7.19	2.70	6	4
			Autotrophic inoculum		7.20	2.70	6.37	3.00	-12	-64
			Soil inoculum		5.21	2.40	4.92	2.70	6	-98
			Uninoculated	With	6.49	3.00	4.52	5.10	6	-86
			Autotrophic inoculum		5.97	2.70	4.92	4.50	1	-98
			Soil inoculum		5.05	3.00	6.54	3.00	12	3

[a]Inhibition is indicated by a negative value, whereas stimulation is indicated by a positive value.

[b]Inoculum taken from a two-day-old culture.

[c]Autotrophic inocula are a mixture of sulfur and ferrous iron oxidizing bacteria.

Table 25. Core and leachate analyses and algal bioassays, core sample DH76-108.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays[a] Static Leachate Percent[b]	Algal Bioassays[a] Shaking Leachate Percent[b]
0- 18	8.46	11.3	Uninoculated	Without	8.56	4.20	6.96	3.90	-54	150
			Autotrophic inoculum[c]		8.48	2.70	7.71	2.40	-53	140
			Soil inoculum		5.70	2.70	7.54	2.10	-74	241
			Uninoculated	With	5.78	2.10	7.51	3.00	-76	195
			Autotrophic inoculum		6.65	3.90	7.65	1.80	38	411
			Soil inoculum		6.22	5.10	7.52	2.10	-32	64
20.3-43.8	8.09	15.0	Uninoculated	Without	7.67	2.70	7.86	1.20	70	61
			Autotrophic inoculum		7.84	2.10	5.98	2.70	113	39
			Soil inoculum		5.42	4.50	6.74	1.80	-59	114
			Uninoculated	With	8.50	2.10	8.55	1.20	43	57
			Autotrophic inoculum		8.18	4.50	8.32	1.50	-16	82
			Soil inoculum		5.67	3.90	7.17	2.70	-42	286

Table 25. Continued.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays Static Leachate Percent	Algal Bioassays Shaking Leachate Percent
75.8-79.2	3.43	11.5	Uninoculated	Without	2.98	5.40	2.88	7.20	-96	-77
			Autotrophic inoculum		2.83	5.10	2.29	4.50	-94	-77
			Soil inoculum		4.71	3.00	4.41	2.10	-77	159
			Uninoculated	With	3.02	2.10	2.76	1.50	-87	-80
			Autotrophic inoculum		3.06	2.70	2.24	2.40	-91	-86
			Soil inoculum		6.03	1.20	4.40	1.20	-46	-45

[a]Inhibition is indicated by a negative value, whereas stimulation is indicated by a positive value.

[b]Inoculum taken from a six-day-old culture.

[c]Autotrophic inocula are a mixture of sulfur and ferrous iron oxidizing bacteria.

Table 26. Core and leachate analyses and algal bioassays, core sample DH75-109.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays[a] Static Leachate Percent[b]	Algal Bioassays[a] Shaking Leachate Percent[c]
22.8-38.9	7.52	15.7	Uninoculated	Without	5.91	0.90	7.63	2.10	-86	-53
			Autotrophic inoculum[d]		5.97	1.20	7.74	2.10	-90	2
			Soil inoculum		6.13	2.10	4.86	3.30	-53	-6
			Uninoculated	With	8.30	1.50	8.08	3.60	-77	1413
			Autotrophic inoculum		8.24	2.40	8.22	3.00	-55	15
			Soil inoculum		6.43	2.40	6.93	2.40	-58	1746
60-114.6	6.06	8.50	Uninoculated	Without	7.36	1.50	8.17	0.30	2	ND[e]
			Autotrophic inoculum		5.93	2.40	7.88	0.90	-3	102
			Soil inoculum		6.26	2.70	4.48	0.30	6	291
			Uninoculated	With	7.93	2.10	7.49	0.90	-46	9
			Autotrophic inoculum		7.93	2.70	6.65	0.30	-50	-7
			Soil inoculum		6.74	2.40	4.61	1.20	0.4	ND

[a] Inhibition is indicated by a negative value, whereas stimulation is indicated by a positive value.

[b] Inoculum taken from a two-day-old culture.

[c] Inoculum taken from a six-day-old culture.

[d] Autotrophic inocula are a mixture of sulfur and ferrous iron oxidizing bacteria.

[e] Not determined.

Table 27. Core and leachate analyses and algal bioassays, core sample DH76-111.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays[a] Static Leachate Percent[b]	Algal Bioassays[a] Shaking Leachate Percent[c]
0-0.5	8.50	57.0	Uninoculated	Without	6.20	1.20	6.13	1.80	-30	136
			Autotrophic inoculum[d]		6.19	2.10	6.24	3.60	-10	255
			Soil inoculum		5.19	0.90	6.42	3.00	-36	84
			Uninoculated	With	8.47	2.70	6.14	2.10	58	132
			Autotrophic inoculum		8.47	0.90	7.08	2.70	22	155
			Soil inoculum		6.00	0.90	6.77	3.30	20	150
13.4-30.5	6.93	15.70	Uninoculated	Without	7.64	2.10	7.77	3.30	-29	355
			Autotrophic inoculum		7.55	0.90	7.80	2.40	-88	150
			Soil inoculum		5.36	1.80	7.31	2.40	-46	86
			Uninoculated	With	7.51	1.80	7.39	2.10	-65	118
			Autotrophic inoculum		7.44	3.30	7.43	1.80	-96	655
			Soil inoculum		5.53	3.60	6.64	3.90	-50	655

Table 27. Continued.

Depth (ft)	Sample pH	Sample Pb (µg/g)	Inoculum Description	GS VI-VII Medium With or Without 0.1% Glucose	Static Leachate pH	Static Leachate Pb (µg/g)	Shaking Leachate pH	Shaking Leachate Pb (µg/g)	Algal Bioassays: Static Leachate Percent	Algal Bioassays: Shaking Leachate Percent
102-105	2.31	20.20	Uninoculated	Without	2.27	2.10	2.09	8.40	-100	-86
			Autotrophic inoculum		2.26	2.10	1.62	10.5	-100	-98
			Soil inoculum		2.71	1.80	1.85	9.00	-100	-93
			Uninoculated	With	2.33	3.00	2.14	5.70	-100	-100
			Autotrophic inoculum		2.18	2.70	1.61	9.30	-100	-98
			Soil inoculum		2.96	3.60	1.95	9.30	-100	-89
131.7-156.5	8.19	17.80	Uninoculated	Without	7.37	3.00	7.16	4.50	-10	-4
			Autotrophic inoculum		7.05	1.20	6.03	2.40	-7	9
			Soil inoculum		4.87	2.40	3.94	6.00	-51	25
			Uninoculated	With	6.79	2.70	5.48	3.90	-7	3
			Autotrophic inoculum		6.35	3.90	4.67	4.50	-10	-13
			Soil inoculum		5.38	0.90	5.71	3.60	-44	-7

[a]Inhibition is indicated by a negative value, whereas stimulation is indicated by a positive value.

[b]Inoculum taken from a two-day-old culture.

[c]Inoculum taken from a six-day-old culture.

[d]Autotrophic inocula are a mixture of sulfur and ferrous iron oxidizing bacteria.

content of the core. The average amount of Pb in the leachates was 2.86 µg/g, with stationary leachates averaging 3.10 µg/g and shaking leachates 2.62 µg/g. The three different leachates, averaging stationary and shaking conditions together, produced average Pb concentrations in the uninoculated flasks of 2.58 µg/g, in the autotrophic inoculum flasks of 2.84 µg/g, and in the soil inoculum flasks of 3.26 µg/g. The addition of glucose produced little changes in Pb values. Specifically, the flasks without glucose were 2.79 µg Pb/g and the flasks with glucose were 2.93 µg Pb/g.

The leaching of DH75-104 resulted in an average Pb concentration of 2.46 µg/g with some leachates going below pH 4.0 (Table 22). Several of the flasks in the 114.4-131.0 ft. sample produced acid, with pH as low as 2.54. The soil inoculum flasks for this sample depth did not produce these low pH values. However, the shaken flasks whether uninoculated or with an autotrophic inoculum produced generally low pH values. The shaken controls also showed higher Pb concentrations than did the stationary controls, 3.96 µg/g and 1.94 µg/g, respectively. When the Pb values for shaken and stationary flasks were combined and arranged by inoculum, the results were: uninoculated 2.43 µg/g, the autotrophic inoculum 2.48 µg/g, and the soil inoculum 2.47 µg/g. The flasks with glucose added to the medium had a composite Pb value of 2.69 µg/g, where the flasks without glucose had 2.27 µg/g.

In Table 23 leachates from DH75-106(A) show similar pH values to the pH values found in other leachates, but lower Pb concentrations. The average Pb content was 1.74 µg/g. The stationary and shaken flasks had average Pb concentrations of 1.31 µg/g and 2.06 µg/g, respectively. The average Pb concentrations from the flasks with the three different inocula were 1.51 µg/g for the uninoculated flasks, 1.71 µg/g for the autotrophic inoculum flasks, and 1.99 µg/g for the soil inoculum flasks. The medium without glucose resulted in an average Pb concentration of 1.47 µg/g, while the medium with glucose resulted in 2.00 µg/g.

In Table 24 core samples from DH75-106(B) are shown to produce some leachates below pH 4.0 and some with Pb concentrations as high as 6.0-6.6 µg/g. Leachates with pH values below pH 4.0 were observed in several of the 201.8-233.4 ft. sample flasks and in the 178-201.8 ft. sample with shaking incubation of the autotrophic inoculum with glucose. The average Pb concentration in the leachates was 3.09 µg/g. The stationary and shaking flasks had Pb concentrations of 3.21 µg/g and 2.97 µg/g, respectively. The inoculum flasks had average Pb concentrations of 2.91 µg/g for the uninoculated flasks, 3.30 µg/g for the autotrophic inoculum flasks, and 3.06 µg/g for the soil inoculum flasks. The average Pb concentration for the different media was 2.77 µg/g when glucose was omitted and 3.41 µg/g when glucose was included.

Core sample DH76-108 in Table 25 had one sample depth, 75.8-79.2 ft., with leachate pH values below 4.0. The leachate from this sample depth, uninoculated, without glucose, and incubated on the shaker, had a Pb concentration of 7.20 µg/g. The average Pb concentrations in the leachates were 3.35 µg/g and 2.52 µg/g, respectively. Inoculum results were 3.05 µg/g for the uninoculated, 3.03 µg/g for the autotrophic inoculum, and 2.70 µg/g for the soil inoculum. Values of 3.41 µg/g and 2.50 µg/g were found for media without and with glucose, respectively.

The leaching of DH75-109 in Table 26 resulted in an average Pb concentration of 1.87 µg/g with no pH below 4.0. Stationary and shaking flasks averaged Pb concentrations at 2.03 µg/g and 1.70 µg/g, respectively. The different inocula had Pb concentrations of 1.62 µg/g for the uninoculated flasks, 1.88 µg/g for the autotrophic inoculum flasks, and 2.10 µg/g for the soil inoculum flasks. The different media, without glucose and with glucose, resulted in 1.65 µg/g and 2.08 µg/g, respectively.

Most sample depths of core DH76-111 (Table 27) resulted in approximately equal pH values. However, sample depth 102-105 ft had pH values below pH 4.0 and Pb concentrations as high as 10.50 µg/g. The shaking autotrophic inoculum flasks of this sample depth had Pb concentrations of 10.5 µg/g and 9.30 µg/g for the without and with glucose media flasks, respectively. The average Pb concentration found in the leachates from this core was 3.36 µg/g. Stationary and shaking flasks had average Pb concentrations of 2.15 µg/g and 4.57 µg/g, respectively. The flasks had average Pb concentrations of 3.16 µg/g for the uninoculated, 2.40 µg/g for the autotrophic inoculum, and 3.53 µg/g for the soil inoculum. The flasks with the different media were very similar in average Pb content with 3.29 µg/g in the without glucose flasks and 3.43 µg/g in the with glucose flasks.

The similarity between the leaching of the different core samples is shown by Tables 20-27. Many samples had a pH drop to below 4.0, while most samples did not alter the pH of the leaching solution appreciably. The pH of the leachates ranged from pH 1.61 to 8.8 (Tables 27 and 24, respectively). Pb concentrations of the leachates ranged from 0.0 µg/g to 10.50 µg/g (Tables 24 and 27, respectively), with the overall average Pb concentration of 2.66 µg/g of core.

Algal Bioassays of Leachates

The results of the algal bioassays for toxicity of leachates from core samples are included in Tables 20-27. The bioassays are reported as percent inhibition, shown by a negative value, or percent stimulation, shown by a positive value. The inoculum of the bioassay experiments was taken from a six-day-old culture or a two-day-old culture; this is noted on each table.

The algal bioassays of the leachates from core DH75-102, using a six-day-old culture as inoculum, are shown in Table 20. Some leachates were toxic, but most leachates showed either no effect or a stimulation of algal growth. Stimulation was as high as 818% for the stationary, soil inoculum flask of sample depth 40-50 ft and 715% for the stationary, uninoculated flask and 535% for the shaking, uninoculated flask of sample depth 80-90 ft. The inhibition of 86% and 98% by the 40-50 ft sample was produced by leachates from the shaken uninoculated and shaken autotrophic inoculum flasks, with pH values of 4.83 and 2.90, respectively, and Pb contents of 2.40 µg/g and 1.95 µg/g, respectively. The other leachates showing inhibition had pH values above pH 6.09 and Pb values below 2.40 µg/g.

Table 21 shows the bioassays from core DH75-103, using a six-day-old inoculum. Most leachates stimulated algal growth with values up to 618% for the shaken, uninoculated, no glucose flask of sample depth 100-110 ft and 162%

for the stationary, autotrophic, with glucose flask of sample depth 10-20 ft. Inhibition was as high as 55% with the stationary, soil inoculum, no glucose flask of sample depth of 100-110 ft. The pH values of the leachates ranged from 5.23 to 8.21 and the Pb concentration ranged from 1.50 µg/g to 4.05 µg/g.

Table 22 shows the results of the algal bioassays of core samples from DH75-104, using a six-day-old culture as inoculum for the stationary leachate bioassays and a two-day-old culture as the inoculum for the shaking leachate bioassays. Stimulation was 139% for the stationary, uninoculated, with glucose flask of sample depth 153.1-169.7 ft and 144% for the shaking, uninoculated, no glucose of sample depth 0-17 ft. However, most of these leachates were toxic, with many having inhibition values of 90%. The leachates of sample depth 114.4-131.0 ft, which had greater than 90% inhibition, had pH values below 4.92 and Pb values ranging from 1.80 µg/g to 5.10 µg/g. The other inhibitory leachates had pH values ranging from 6.57 to 7.80 and Pb values from 0.60 µg/g to 3.60 µg/g. The leachate of the stationary, uninoculated, without glucose flask from sample depth 200.1-219.5 ft had a pH value of 6.97 and a Pb content of 0.60 µg/g and produced 9% inhibition. The leachate of the shaking, autotrophic inoculum, without glucose flask from sample depth 153.1-169.7 ft had a 98% inhibitory effect.

Table 23 gives algal bioassays of leachates from core DH75-106(A). Stationary leachates used a six-day-old inoculum and the shaking leachates a two-day-old inoculum. Stimulation was 650% for the stationary, uninoculated, with glucose flask of sample depth 10-11 ft, and 335% for the stationary, soil inoculum, without glucose flask of sample depth 5-10 ft, and 297% for the shaking, uninoculated, without glucose flask of sample depth 40 ft. The pH values and Pb concentrations for these flasks showing stimulation were pH 8.58 and 1.20 µg/g, pH 5.51 and 2.0 µg/g, and pH 8.44 and 2.10 µg/g, respectively. The inhibition by these core leachates ranged from 1% to 91%. The leachates showing inhibition had pH values ranging from 5.82 to 8.52, and Pb content ranging from 0.90 µg/g to 4.20 µg/g.

The bioassays for core DH75-106(B) (Table 24), using a two-day-old inoculum, showed less stimulation than that seen in the other cores. The highest stimulatory effect was 83%. The pH range was 5.05 to 8.80 and the Pb content ranged from not detectable to 5.40 µg/g. Some flasks totally inhibited (100%) algal growth. Most flasks from sample depth 201.8-233.4 ft showed greater than 90% inhibition. The pH of these leachates was 2.31 to 4.41 and the Pb varied from nondetectable to 6.60 µg/g. Inhibition was observed with leachates of pH range 2.31 to 8.41 and at all measured lead concentrations.

The bioassays of core sample DH76-108 in Table 24 used a six-day-old inoculum. Stimulation was as great as 411% in the case of the shaking, autotrophic inoculum with glucose flask of sample depth of 0-18 ft, at a pH of 7.65 and a Pb content of 1.80 µg/g. Stimulation was also seen when the leachate was pH 4.41 to pH 8.50 with Pb content of 1.20 µg/g to 3.90 µg/g. Inhibition was observed in leachates of pH 2.24 to 8.56 and of Pb content of 1.20 µg/g to 7.20 µg/g. The leachate from sample depth 75.8-79.2 ft had inhibition of 45% to 96%, a pH range of pH 2.24 to pH 6.03 and a lead content of 1.20 µg/g to 7.20 µg/g.

Table 26 shows the algal bioassays of core DH75-109, using a two-day-old culture for the stationary leachates and a six-day-old culture for the shaking leachates. Stimulation was 1413% and 1746% in the shaking, with glucose flask of both the uninoculated and soil inoculum from sample depth 22.8-38.9 ft, respectively, which had pH's of 8.08 and 6.93 and Pb concentrations of 3.60 µg/g and 2.40 µg/g, respectively. Leachates showing stimulation had a pH range of 4.48 and 8.22 and a Pb content range of 0.90 µg/g to 3.60 µg/g. Inhibition was observed with leachates with a pH range of pH 4.86 to pH 8.30 and with a Pb range of 0.30 µg/g to 2.70 µg/g.

Algal bioassay results of leachates from core DH76-111 are seen in Table 27. A two-day-old culture was used as inoculum for the stationary leachate bioassays, while a six-day-old culture was used for the shaking leachate bioassays. Stimulation was as great as 655% in the bioassays, specifically the autotrophic and soil inocula of the shaking, with glucose flasks which had pH values of 7.43 and 6.64 and Pb concentrations of 1.80 µg/g and 3.90 µg/g, respectively. Inhibition, as seen in all of the flasks of sample depth 102-105 ft, often exceeded 86%. The leachates from sample depth of 102-105 ft had a pH range of pH 1.61 to pH 2.96 and a Pb content range of 1.80 µg/g to 10.50 µg/g. Inhibition was seen from leachates of the pH range, pH 1.61 to pH 7.64 and of Pb content range, 0.90 µg/g to 10.50 µg/g.

The algal bioassays of the leachates resulted in stimulation of as high as 1746% and in inhibition of 100%. The pH and Pb content ranges of the leachates showing stimulation were pH 3.94 to pH 8.80 and 0.00 µg/g to 6.00 µg/g. The ranges of pH and Pb content for those leachates showing inhibition were pH 1.61 to pH 8.56 and 0.00 µg/g to 10.50 µg/g. The extreme values for a six-day-old culture were 1746% stimulation and 100% inhibition. The extreme values for a two-day-old culture were 297% stimulation and 100% inhibition.

The algal bioassays of the low pH leachates, when adjusted to pH 8.0 with 0.1 N NaOH, are seen in Table 28. The leachates were originally pH 2.18 to pH 5.10 with lead contents from below the detectable limit to 6.00 µg Pb/g. Raising the pH to 8.0 decreased the toxicity of 42% of the leachates by 30%. Another 46% of the leachates became less toxic by 1 to 22%. But the remaining 12% of the leachates became more toxic by 36 to 60%.

Relation of Chemical Composition to Presence of Bacteria

After completion of the leaching studies, an attempt was made to correlate chemical composition of the cores with presence of iron and sulfur oxidizing bacteria. Eight samples were selected from cores that had yielded cultures of these bacteria and six from cores in which these bacteria were not detected. The samples chosen were not exact replicates of those used for the bacterial studies since those were no longer available; the samples were taken from adjacent sections of core. Eight chemicals were determined and the results are shown in Table 29. Of the two samples with the highest iron content, one was from a core having iron oxidizing bacteria and one was not. The same was true for sulfur. Moreover, when the cores were divided into two groups, one positive for bacteria and the other negative for bacteria, the mean and standard deviation of each element were not significantly different

Table 28. Results of algal bioassays of leachates of core samples following pH adjustment to pH 8.0, percent stimulation or inhibition.[a/]

Core sample	Depth (ft)	Inoculum description	GS VI-VII medium with or without 0.1% glucose	Stationary leachate[b/]	Shaking leachate[b/]
DH75-102	40-50	Uninoculated	With		-15
		Autotrophic inoculum			-64
DH75-104	114.4-131.0	Uninoculated	Without		+355
		Autotrophic inoculum			-52
		Soil inoculum			-70
		Uninoculated	With		-66
		Autotrophic inoculum			-57
		Soil inoculum			-59
DH75-106(B)	178-201.8	Autotrophic inoculum	With		-65
	201.8-233.4	Uninoculated	Without	-67	-98
		Autotrophic inoculum		-53	-98
		Soil inoculum		-81	-77
		Uninoculated	With		-98
		Autotrophic inoculum			-91
		Soil inoculum			-74
DH76-111	102-105	Uninoculated	Without	-99	
		Autotrophic inoculum		-47	
		Soil inoculum		-90	
		Uninoculated	With	-99	
		Autotrophic inoculum		-67	
		Soil inoculum		-99	

[a]Bioassays with leachates from core samples DH75-102 and DH75-104 used a six-day-old inoculum, while bioassays with leachates from core samples DH76-111 and DH75-106(B) used a two-day-old inoculum.

[b]Inhibition is indicated by a negative (-) value, whereas stimulation is indicated by a positive (+) value.

Table 29. Chemical analysis of overburden core samples in relation to presence of bacteria which oxidize iron and sulfur compounds.

Core Sample No.	% $CaCO_3$ (Equiv.)[b]	Chemical Analysis[a] (µg/g) As	B	Cd	Fe	Pb	Hg	Se	S[c]
Cores with Bacteria Present									
104-4	11.7	1.8	9.5	0.33	439	9.9	0.056	0.21	0.05
104-11	2.0	3.4	5.4	0.36	301	10.9	0.060	0.12	0.03
104-17	12.4	5.1	22.3	0.73	246	9.4	0.213	0.69	1.08
106B-9	1.6	2.2	9.8	0.44	653	13.1	0.066	0.32	0.05
108-1	11.0	8.2	5.1	0.39	769	15.0	0.055	0.38	0.18
108-3	7.1	8.3	5.1	0.48	951	24.2	0.110	0.38	0.16
109-3	12.3	4.0	3.2	0.58	572	6.7	0.025	0.065	0.27
111-1	9.9	5.7	5.9	0.27	485	42.0	0.080	0.16	0.01
$\bar{x}$[d]	8.5	4.84	8.29	0.45	552	15.4	0.08	0.29	0.23
s[e]	4.47	2.48	6.10	0.15	236.28	11.62	0.06	0.20	0.36
Cores with Bacteria Absent									
102-6	8.7	10.8	7.5	0.52	565	65.5	0.197	0.3	0.33
103-10	1.1	1.4	1.7	0.31	139	7.1	0.060	0.14	0.04
104-6	11.9	2.8	13.9	0.39	504	10.4	0.054	0.20	0.01
104-16	8.1	2.7	3.7	0.38	161	11.0	0.072	0.31	0.11
109-1	9.7	5.0	4.3	0.33	559	10.9	0.050	0.40	0.22
109-2	12.3	8.1	3.6	0.36	1219	15.6	0.042	0.29	0.46
$\bar{x}$[d]	8.63	5.13	5.78	0.38	524.5	20.08	0.08	0.27	0.20
s[e]	4.06	3.64	4.40	0.07	391.49	22.41	0.06	0.09	0.18

[a] All elements are acid digested and reported "Total".

[b] Method performed: Agriculture Handbook #60 USDA, Method 23C. This value usually is somewhat high because soil constituents other than lime may react with acid.

[c] Sulfur reported as percent total content.

[d] Mean value all samples.

[e] Standard deviation.

for the two groups. The table does give additional data supporting the heterogeneity of the overburden. It also suggests that such an analysis should precede leaching studies since some samples had much higher values of potentially toxic elements than other samples. It should be remembered that the iron and sulfur oxidizing bacteria which were obtained in enrichment culture in this study were not typical chemolithotrophs and had unidentified nutritional requirements. For this reason, the lack of correlation of bacteria and chemical analysis of the cores is not too surprising.

SECTION VII

DISCUSSION

The solubilization of metals from mining spoils, including coal mine spoils, by microorganisms has been well reviewed by Silverman and Ehrlich (1964), Tuovinen and Kelly (1974), Dugan (1975) and most comprehensively by Brierley (1978). This leaching of mineral occurs through the oxidation of a substrate, inorganic or organic. Sulfide minerals produced acid end products (Brierley 1978). Many microorganisms have been studied in relation to their production of acid and the resulting solubilization of metals. These microorganisms include the chemolithotrophic *Thiobacillus* species (Bryner and Jameson 1958; Galbraith et al. 1972; Leathen et al. 1953a; 1953b; Temple and Delchamps 1953) and a thermophilic *Sulfolobus*-like bacterium (Brierley 1978), also heterotrophs such as the fungus *Penicillium simplicissimum* (Silverman and Munoz 1971), and the bacterium *Bacillus* (Tuovinen and Kelly 1974). Heterotrophs definitely enhance leaching but at a lower order of magnitude than chemolithotrophs. *Thiobacillus* species play an important role in the solubilization of several metals by greatly accelerating the oxidation of metal sulfides in mineral and sulfide-bearing coal deposits through acid and ferric iron production (Silverman and Ehrlich 1964; Temple and Delchamps 1953). The rate and amount of observable leaching is influenced by the composition of the deposit, for example, by the amount of pyrite or carbonate present (Dugan 1975; Sokolova and Karavaiko 1964). The toxicity of ground water containing high acid and/or metal concentrations is therefore dependent upon the overall composition of the strata through which the ground water percolates (Dugan 1975).

The cursory analysis of the core samples from the West Moorhead coal field showed a great diversity in the overburden composition. A wide variety of sedimentary deposits was visually apparent. A diverse chemical composition was evident from the pH, conductivity, and Pb analyses. A general relationship between the composition of a natural water and the strata with which the water has been in contact is certainly to be expected (Hem 1970).

The pH values of the core sample slurries encompassed a wide range (pH 2.31 and 9.65 in Tables 10 and 11). The pH values in the water percolating through the particular zone of each sample would be affected by these measured values. But ground water, having percolated through many overburden strata would reflect a composite effect of the different pH's in these strata. The ground water would therefore not exhibit such a wide pH range; e.g. pH values range from 5.3 to 8.4 in ground water of the West Moorhead coal field (unpublished data, U.S. Geological Survey, Billings Division). Localized areas in the cores demonstrating low pH values, below pH 5.0, could be useful

sources of acidophilic chemoautotrophic bacteria and would be appropriate for any subsequent leaching study. Low pH values in spoils may be due to the presence of metal sulfides, pyrite being the commonest, which on exposure oxidize to the corresponding metal sulfate and sulfuric acid (Fjerdingstad et al. 1976; Silverman and Lundgren 1959; Temple and Delchamps 1953). This acid production effectively lowers the pH of the sample and ground water passing through it. The actual pH depends upon the buffering capacity of minerals in the sample, the buffering capacity of the water, and the amount of acid produced from the sample.

Conductivity of the core samples was employed as an easily measured, general indication of the ion concentration, although conductance can not be expected to be simply related to ion concentration without a thorough analysis of the samples (Hem 1970). From the conductivity values observed, one could postulate that the general inorganic nutrient level in the core would support a large chemolithotrophic population and be more than adequate for heterotrophs (which would be limited by the supply of organic compounds). The conductivity readings could also reflect the acid salts resulting from the neutralization of bacterially produced acid by the carbonates in the overburden.

Lead was tested as an easily measurable parameter of metal composition and as a possible indicator of autotrophic leaching, not with the idea that high levels were present. According to the analyses, small quantities of Pb were present, in the µg/g of core range, and concentrations were variable in this range. The solubility of Pb, whether present in the core as a sulfide, hydroxide, or carbonate, would be affected by acid concentration in the leach water. If leaching in the core samples was due to acid production, lead concentration in the leachate would be a rough qualitative indicator of this.

Although the West Moorhead coal and the Fort Union coal in general are composed of low-sulfur subbituminous and lignite coals (Matson and Blumer 1973), considerable variation exists. Chadwick et al. (1975) found vertical distributional variations of trace elements and sulfur, probably dependent upon delicate changes in the geological and chemical environment after consolidation of the coal. Chadwick et al. (1975) also noticed a sulfur enrichment in the basal footage of the coal seams related to visible concentrations of pyrite and other metal sulfides in vertical fractures of the coal. The overburden exhibits even greater variability. Our field observations in the Colstrip area of the Fort Union coal field show masses of nodular pyrite and also of large pyrite crystals. These are sporadically distributed in surface spoils and must reflect a marked variability in pyrite concentration in the overburden at that locality. The bicarbonate level of the ground water from overburden and coal is relatively high, suggesting high carbonate levels in the overburden strata of the West Moorhead coal field (unpublished data, U.S. Geological Survey, Billings Division). The alkalinity of the ground water can be explained from the low sulfide and/or high carbonate concentrations in the Fort Union coal deposit and its overburden. There may be no relation between the amount of metal sulfide leached and metal concentration in the ground water, due to buffering, neutralizing and precipitation due to carbonates in the strata and to bicarbonate in the ground water.

Thiobacillus species are associated with coal mines and coal mine drainages (Dugan 1975; Tabita et al. 1970). The extremely acidophilic species are

often numerous in coal mines where they grow in drainage channels, on moist surfaces of pyrite in the coal face and on moist pyrite in the floor and roof strata after these are exposed to air and water by mining (Dugan 1972; Dugan 1975; Temple and Delchamps 1953). The extremely acidophilic *Thiobacillus* species include *T. thiooxidans* and *T. ferrooxidans*. The spoils, from which large quantities of acid are produced, have a high sulfur content with pyrite as the predominant sulfide mineral. When the spoils contain large quantities of carbonates, the titratable adicity, total salts, sulfate and dissolved iron in the mine drainage all decrease (Torma et al. 1970). Along with this apparent decrease in acid production is an increase in population of other *Thiobacillus* species, especially *T. intermedius* and *T. thioparus* (Leathen et al. 1953a; Parker 1947; Tabita et al. 1970). Karavaiko (1961) found *Thiobacillus thiooxidans* in high carbonate sulfur deposits, but not until he analyzed for their distribution in microzones, instead of in bulk samples. Olson (1978) found *T. ferrooxidans* in alkaline coal strip mine drainage in numbers that were equal to those found in certain acid mine drainages. Studies of strata producing alkaline drainage indicate the presence of *Thiobacillus* species, which are associated with acid drainage, but their acid producing activity is masked by the alkaline composition of the strata (Karavaiko 1961; Olson 1978).

The present study indicated a wide distribution of chemoautotrophic sulfur and iron oxidizing bacteria probably belonging to the genus *Thiobacillus*, in the overburden cores from the high carbonate coal fields of eastern Montana (Tables 12-19). It would be possible to implicate a large number of the *Thiobacillus* species on the evidence of the percent thiosulfate oxidized and the resulting pH in the enrichment cultures on the media of Hutchinson et al. (1965, 1966, 1969). Our results do not implicate the two extremely acidophilic species. Karavaiko (1959, 1961) found microzones in the high carbonate sulfur deposit with high populations of *T. thiooxidans* and with pH values less than pH 4.0. Unfortunately, the micromethods of Karavaiko (1961) were not employed in these studies. However, *T. ferrooxidans* was isolated from a revegetation site (1969-11) at Colstrip, Montana. It oxidized 99% of the ferrous iron and produced a final pH of 1.84. This isolate was similar to the *T. ferrooxidans* cultures isolated from alkaline ground waters of the Decker coal field by Olson (1978).

Purification of the chemoautotrophic enrichments from the overburden cores were unsuccessful due to failure of the bacteria to grow adequately. Thiosulfate cultures grew on repeated transfers in liquid medium and also on transfers from liquid medium and solid medium to solid medium. However, transfer of well isolated colonies to fresh solid medium resulted in multiple colony type formation, while their transfer to liquid medium would not result in growth. When yeast extract was included in the liquid thiosulfate medium, transfers of cultures growing in liquid medium produced growth, but transfers from well isolated colonies again did not produce growth.

The purification of the ferrous iron cultures from the overburden cores was also unsuccessful. The bacteria failed to grow on repeated transfers in liquid media, even when yeast extract, cysteine, glutathione, or IM-MF additives were included in the iron medium. To place these failures in perspective: the well described and recognized species of thibacilli grow readily in laboratory media. However all researchers on this group encounter strains which

grow only in liquid media, not on agar. Some strains can be subcultured only in the presence of other organisms. Brierley and others (private communication) have found strains which respond well to yeast extract, to cysteine and to other organic supplements but there are still unsolved problems in the cultivation of many bacteria with chemolithotrophic properties.

The algal bioassay, following a procedure of the Environmental Protection Agency (1971), has been used to manage water quality, to evaluate the fertility of water, and to determine the toxicity of inorganic or organic compounds (Greene et al. 1975; Payne 1975; Miller et al. 1978). The U.S. EPA (1971), and Miller et al. (1978) recommend the use of *Selenastrum capricornutum* Printz, a green alga (Chlorophyceae) of the order *Chlorococcales*, as the test alga. *Selenastrum* belongs to the group of ubiquitous algae which include *Chlorella*, *Scenedesmus*, and *Ankistrodesmus*, which have a wide tolerance toward environmental conditions (U.S. EPA 1978). The parameter used to describe growth of the test alga is maximum standing crop, which is defined as the maximum biomass achieved during incubation, but for practical purposes is assumed to be obtained whenever the increase in biomass is less than 5% per day (U.S. EPA 1971; Miller et al. 1978). Biomass can be monitored by several methods, which include dry weight (gravimetric), dry weight (indirect electronic particle counting), chlorophyll *a* (*in vivo* fluorescence, extracted fluorescence and extracted absorbance), direct microscopic enumeration, and absorbance (turbidity at 750 nm)(U.S. EPA 1971; Miller et al. 1978).

All of the above methods except dry weight were compared to determine which would be the simplest reliable method under our conditions. The three methods of monitoring cholorphyll *a* all reached maximum standing crop, whereas the other monitoring methods did not. *In vivo* fluorescence was selected as the method to measure chlorophyll *a*, due to the simplicity and rapidity of the method.

Algal growth curve studies were performed to determine the influence of pH, glassware acid washing, inoculum size, inoculum age, and inoculum washing (Fig. 3). These studies were undertaken to improve the reproducibility of the growth curves in the algal bioassay controls inoculated from six-day-old cultures of the alga (Fig. 4). From the curves seen in Figure 2, glassware acid washing, inoculum size, and inoculum washing did not affect the maximum standing crop obtained. All pH values less than 5.3 were toxic to the alga. A two- or four-day-old inoculum, instead of the six-day-old inoculum, approximately doubled the maximum standing crop. The transfer of algal cultures on two-, four-, or even six-day intervals was not consistent with the seven-day interval recommended by the EPA, but resulted in the production of a more continuously logarithmic culture which also gave more reproducible results [Fig. 4 (U.S. EPA 1971, 1978)].

Leaching of the overburden core samples was performed with samples ground to <80 mesh, or <117 μm. Particle size influences the rate of leaching (Bryner and Anderson 1957; Temple and Delchamps 1953). The particle size used was consistent with that used in successful microbiological leaching of copper and molybdenum minerals [particle sizes ranging from <60 mesh (Bryner and Anderson, 1957) to 63 to 200 μm (Bosecker et al. 1978)]. There were three inoculum conditions, uninoculated, and autotrophic inoculum, and a soil inoculum. The

uninoculated flask allowed leaching by the natural bacterial population of the core samples. The autotrophic inoculum was a mixture of sulfur and iron oxidizing bacteria [as was the inoculum Bosecker et al. (1978) used in metal leaching experiments]. The soil inoculum was included to represent the effect of an actively growing heterotrophic bacterial and fungal contingent. Glucose was not included in all of the leach media, since organic compounds can have inhibitory effects on autotrophic growth (Lundgren et al. 1972). These different incubation procedures were used, not in order to compare them with each other, but in the hope that one or more of them would yield an interesting leachate.

The present study used pH and Pb concentration of the leachate to evaluate the leaching in overburden core samples. The leachates were also examined by algal bioassay to determine their potential toxicity, which could be due to low pH, various metal concentrations, or other factors. The fact that most leachates did not show an appreciable pH decrease is almost certainly caused by the high carbonate concentrations found in the overburden of the West Moorhead coal field (Matson and Blumer 1973). Some samples did yield acid leachates, with pH values as low as 1.61 (Table 27). The Pb values in the leachates were roughly similar and not proportional to the Pb content of the core samples. This strongly implies that autotrophic oxidation did not proceed at a rate associated with unbuffered high pyrite ores.

Toxicity was observed in the algal bioassays of some leachates. Some leachates had a stimulatory effect on algal growth, and others had no effect. This toxicity is readily explained by the low pH in the case of some leachates. However, toxicity was also seen with leachates of high pH, as well as with low pH leachates after the pH had been adjusted to 8.0. Lead concentrations could conceivably influence the observed toxicity, but no relationship between total soluble Pb concentrations and algal growth inhibition was observed. Moreover, the Pb concentration in the leachates was probably well below toxic levels and there was a ten-fold dilution of the Pb in the bioassays. Aside from some acid leachates, toxicity was not explained by the limited analyses of leachates in this study.

Chemoautotrophic bacteria were widely distributed in the overburden cores from the Bear Creek Study Site of the West Moorhead coal field in the Fort Union coal deposit located in eastern Montana. This group of bacteria was also found in the drainage from the Decker coal strip mine, also located in the Fort Union coal deposit in eastern Montana (Olson 1978). According to our understanding of the physiology and nutrition of these bacteria, they do not belong among the facultative autotrophs but grow only at the expense of an inorganic energy source. There is an element of uncertainty in this conclusion. But the odds are that they are producing acid in these core strata, which if true requires the conclusion that leaching in these overburden cores is accelerated by this bacterial action. However, in this study most strata did not develop low pH leachates, which, along with the widespread occurrence of chemoautotrophic bacteria, means (a) there is a relatively small amount of acid-producing sulfide present or (b) there is a relatively large amount of neutralizing mineral notably carbonates in most strata. This study has not defined which of these explanations is the case, although Matson and Blumer (1973) show a low sulfide and a high carbonate level in strata in this area. If toxic elements

are present, their leaching should be accelerated by the action of the chemo-autotrophic bacteria whose presence has been demonstrated. Toxicity studies on our leachates, using an algal bioassay, show several effects. Some leachates had a stimulatory effect on algal growth, suggesting an unexplained eutrophication of the receiving water. Other leachates produced an inhibitory effect on algal growth which was explained by the low pH of these leachates. Inhibition, which was reproducible, was observed with other leachates, but was not explained by the data gathered during this study. All of this suggests that the potential for acid pollution does exist in eastern Montana, but that its development into a pollution problem would be sporadic and possibly minor.

SECTION VIII

SUMMARY

Eight test hole cores of overburden grab samples from eastern Montana coal fields were received from the U.S. Geological Survey. The samples were visually inspected for evidence of mineralization, aided by an accompanying geological log for each core. Portions of samples were examined for lead content, pH, conductivity, and the presence of chemoautotrophic bacteria. The analyses of core samples, given in Tables 4-11, show quantitatively different mineralization in the strata and conductivity values varying over a wide range. These results had no consistent relation to the physical description of the strata and could not be anticipated by a visual examination of the cores. The pH values encompassed a much wider range than either Pb or conductivity.

Chemoautotrophic sulfur and iron oxidizing bacteria were isolated from a number of the core samples. Difficulty was encountered in obtaining pure cultures of these bacteria on solid media. The bacteria differed from other fastidious iron oxidizers isolated by J. Brierley (private communication).

A culture of iron oxidizing bacteria was isolated from a revegetation study site (1969-11) at Colstrip, Montana which exhibited evidence of iron oxidation in close association with coal distributed in the overburden. This culture was similar to the typical iron oxidizing bacterium, *Thiobacillus ferrooxidans*.

Leaching studies produced a wide range of lead concentrations, pH values, and conductivity values. Leach media were inoculated with either a soil mixture obtained from greenhouse pots or a mixture of autotrophic bacteria obtained from various sources, or were uninoculated. Lead values in the leachates were roughly similar and not proportional to the Pb content of the core sample. Autotrophic oxidation did not proceed at the rapid rate associated with unbuffered high pyrite ores.

Most strata did not develop low pH leachates; a few did (Table 22).

Algal bioassay results (Tables 20-27) showed growth inhibition by some leachates. The toxicity effect in some cases was explained by low pH values. Other toxic leachates could not be explained by the data. For the majority of leachates, no toxicity was observed, instead some leachates produced stimulatory effects.

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